STUDIES ON NUTRITIONALLY DEFICIENT STRAINS OF *PSEUDOMONAS AERUGINOSA*

1. THE PRODUCTION BY X-RAYS AND THE ISOLATION OF NUTRITIONALLY DEFICIENT STRAINS

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The irradiation of *Neurospora crassa* with X-rays by Beadle and Tatum (1941) and their isolation of strains lacking in ability to synthesize certain growth factors stimulated the search for similar deficiencies in bacterial cells. Gray and Tatum (1944) obtained deficient strains of *E. coli* and *Acetobacter melanogenum* by treatment with X-rays. Their strains of *E. coli* required the addition of biotin and threonine while their strains of *Acetobacter* required glycine or serine, and adenine or adenosine. Roepke, Libby and Small (1944) isolated eight deficient strains of *E. coli* following X-ray treatment but since they also obtained four deficient strains from their unirradiated control they were not fully satisfied that the X-rays were responsible for the greater number isolated following irradiation. Roepke and Mercer (1947) isolated a further 29 deficient strains from 4,420 colonies tested after X-ray treatment.

Tatum (1945) using deficient strains previously isolated, obtained, by a second period of irradiation, strains requiring the addition of two separate factors to the basal medium.

E. coli has been used almost universally for studies of the action of X-rays on bacteria because of its simple growth requirements and the ease with which deficient strains can be isolated. This paper deals with the production and isolation of nutritionally deficient strains from Pseudomonas. This organism was selected because of its simple growth requirements and because of the general resistance of Pseudomonas strains to various antibacterial agents. The nutritionally deficient strains were to be used for the study of this general resistance.

Materials and Methods.

The organism.

The strain (P95) of *Pseudomonas aeruginosa* was isolated by French (1947) and a single cell was obtained from this strain by the method of K. I. Johnstone (1943). All

work was performed on cultures derived from this single cell, and cultures which had been subcultured more than four times from this original cell were discarded.

Growth requirements of the parent strain.

The growth requirements of this strain were determined by incorporating both carbon and nitrogen requirements at a concentration of 0·3 p.c. when supplied independently, and also at 0·3 p.c. when they were supplied by a single substance in doubly-glass-distilled water. Salts and trace elements were incorporated at the same concentrations used by Williams (1942) for microbiological assay.

Doubly-glass-distilled water was used to prepare M/20 solutions of the more soluble growth factors and M/200–M/1,000 solutions of the more insoluble ones.

The irradiation of the parent strain.

The organisms were grown in nutrient heart broth enriched with 0·5 p.c. Difco yeast extract. They were centrifuged from this enriched broth while still in the logarithmic phase of growth. The sediment of organisms was suspended in normal physiological saline, standardized by turbidity using a Spekker photoelectric absorptiometer and diluted to give 100×10^6 organisms per ml. Two ml. of this suspension were irradiated in a 6 in. × ⅝ in. Pyrex test tube with a small bulb at the bottom. The suspension was stirred mechanically with a glass stirrer during irradiation. The total time of irradiation was controlled so that the number of survivors was suitable for the particular isolation technique to be used.

The organisms were exposed to rays from:

(a) a Philips 6 KW heavy anode tube operated at 40 kV and 4 mA using an Engeln Portable X-ray Apparatus Type F, (b) a Philips Contact Therapy unit operating at 50 kV and 2 mA, (c) a Watson Victor Superficial Therapy Unit KX 140 which was operated at 110 kV and 5 mA. This unit had an SP 140 tube.

Isolation of nutritionally deficient strains after irradiation.

Method 1. The first method of isolation consisted of plating out the irradiated suspension on enriched nutrient agar and incubating for 24 hours at 37° C. A number of the colonies resulting from the survivors were then picked off into 10 ml. of sterile distilled water, from which two tubes were inoculated, one containing a basal medium of α alanine and the other enriched broth.

Method 2. The second method followed the work of Lederberg and Tatum (1946) who developed a technique whereby survivors were incorporated in a basal agar and incubated for 24 hours. Any colonies which developed were marked and the medium then enriched either by addition of enriched broth by diffusion from small glass cylinders fused on to the agar or by pouring a layer of nutrient agar over the surface. In the present work the irradiated suspension was spread over the surface of well-dried α alanine basal agar plates and growth factors were added in the glass cylinders.

Method 3. The third method involved a preliminary period of incubation in enriched broth. Demerec (1946) found that such an incubation was necessary for the phenotypic expression of some mutants following X-ray treatment. Method 3 therefore consisted of method 2 with six hours' incubation in enriched broth prior to plating out on the basal agar. The cells were centrifuged and washed before plating out. α alanine, asparagine and glucose-ammonium sulphate agar were all used as basal media. The modification introduced here was used in all subsequent methods.

Method 4. This was a modification of that devised by Davis (1948) and Lederberg and Zinder (1948) working independently and was based on the action of penicillin which steri-

lized only those cells which were actively respiring. The method included a period of incubation in a basal medium in the presence of an inhibitory concentration of penicillin. In the present work the cells after washing were incubated in a glucose-ammonium sulphate medium (containing 10,000 units of penicillin per ml.). Samples were removed at 17, 21 and 24 hours and plated on to enriched agar plates. Instead of picking off the colonies into water they were touched with a sterile loop which was then used to inoculate a basal agar plate and an enriched agar plate. This variation facilitated the handling of large numbers of colonies. Care was taken not to disturb the agar when touching the colony.

Method 5. This was used to isolate further deficient strains from five strains, already deficient, by irradiating a second time. This method consisted of method 4 with the modification that the specific growth factors which the organism was known to require were added to the basal medium before the penicillin treatment.

The nutritional requirements of the isolated strains.

Cells were picked off agar slopes kept in the cold and suspended in sterile distilled water. This suspension was then seeded into basal agar plates to give a final concentration of about 10^6 organisms per ml. Growth factor mixtures were prepared containing six to eight substances mostly at about M/100 concentration, but the more insoluble ones at M/1,000 to M/5,000. Five different mixtures were used containing most of the amino acids, vitamins and some nucleic acid fractions. These solutions were added to the basal agar by diffusion from small glass cylinders fused on the agar surface. Stimulation of growth around a particular cylinder after 24 hours' incubation at 37° C. indicated that substances necessary for growth were contained in that solution. The constituents of that solution were tested individually for growth stimulation by the same method used for the original mixtures.

RESULTS.

Growth requirements of the parent strain.

α alanine at 0·3 p.c. in distilled water was the simplest compound capable of supplying both the carbon and nitrogen for the parent strain. The organisms also grew when supplied with asparagine at 0·3 p.c. or glucose and ammonium sulphate also at 0·3 p.c. Growth was stronger on the asparagine medium than on the other two.

Isolation of nutritionally deficient strains after irradiation.

Method 1. About 200 colonies could be examined for each irradiation. Over 4,000 colonies were investigated following 23 irradiations but no nutritionally deficient strains were isolated. Some strains were obtained which required longer than 24 hours to show visible growth in the basal medium. This delay always disappeared on subsequent subculture.

Method 2. About 2,000–4,000 colonies could be examined by this technique after each irradiation. About 25,000 colonies were investigated following eight irradiations and no nutritionally deficient strains were isolated. However, a few strains with a temporarily delayed growth in the basal medium were isolated.

Method 3. Following 10 irradiations more than 30,000 colonies were investigated. From each irradiation 200–300 survivors were examined. Their number was increased about 10-fold by the six-hour period of incubation and thus 2,000–4,000 colonies were examined per irradiation. Only about 3,000 direct survivors were thus examined from the ten irradiations. Suspensions from four experiments were tested on α alanine agar, from three on asparagine agar, and from three on glucose-ammonium sulphate agar. Once again no deficient strains were isolated but strains showing a temporarily delayed growth in the basal media were isolated.

Method 4. About 20,000 survivors could be handled following one irradiation and nutritionally deficient strains were isolated by this method. One to four distinct types were isolated per irradiation, i.e. per 20,000 survivors. When the preliminary period of incubation in a complete medium outlined in Method 3 was omitted in three experiments no deficient strains were isolated. Eight irradiations yielded 40 nutritionally deficient colonies. All the 40 colonies were examined but only nine different nutritional deficiencies were found. These are shown in the first column of Table 3. The 15 strains shown in Table 1 were selected as representatives of the different deficiencies isolated following each of the irradiations.

No deficient colonies were isolated from 10^6 cells treated without a period of irradiation.

Method 5. Several doubly deficient strains were isolated using the modification of adding the factors which they originally required to the basal medium before penicillin treatment. Five strains already deficient were each irradiated once. Thirty colonies with additional deficiencies were obtained. Two new nutritional deficiencies were found, but eight of the previously isolated deficiencies occurred again. From this work ten new strains were isolated. They were different from all previous strains because they contained double deficiencies and not necessarily because any new deficiencies had appeared. (See Table 3.)

The nutritional requirements of the isolated strains.

The results of the investigation of the requirements using mixtures of growth factors are set out in Tables 1 and 2. Table 1 shows the strain number and the effect of the five mixtures of growth factors on each strain.

Table 2 deals with the determination of the growth requirements of strains irradiated for a second period.

The individual requirements of the strains were determined by testing each individual substance of the stimulating mixture in the same way as the mixtures. Table 3 shows the requirements of the strains following a single and double period of irradiation and the numbers of the strains with that particular requirement.

TABLE 1.

The effect of the addition of various growth factor mixtures to a basal medium on strains isolated following a single period of irradiation.

Strain No.	Mixtures of growth factors. A	B	C	D	E
2	—	+	—	—	—
4	+	+	—	—	—
14	—	+	+	—	—
16	—	—	—	—	+
19	—	—	—	+	—
20	—	—	+	+	—
22	—	—	—	+	—
24	—	+	+	—	—
25	—	—	+	—	—
28	+	+	—	—	—
31	—	+	+	—	—
32	—	—	—	+	—
33	—	+	+	—	—
36	—	—	+	—	—
39	—	—	—	+	—

\+ indicates growth stimulation.
— indicates no growth stimulation.

The mixtures A, B, C, D and E contained the following substances:

A. Nicotinic acid, calcium pantothenate, riboflavin, pyridoxine, thiamine and biotin.
B. Xanthine, uracil, thymine, guanine, adenine, inositol and *p.* aminobenzoic acid.
C. Histidine, tyrosine, arginine, phenylalanine, proline and glutamic acid.
D. Glycine, alanine, tryptophane, lysine, leucine, isoleucine, and valine.
E. Methionine, cysteine, cystine hydrochloride, hydroxyproline, serine, and threonine.

TABLE 2.

The effect of the addition of various growth factor mixtures to a basal medium on strains isolated following a second period of irradiation.

Strain[1] No.	Irradiated[2] Strain No.	Mixtures of growth factors. A	B	C	D	E
40	16	—	+	+	—	—
41	16	—	+	—	—	—
43	16	+	—	—	—	—
44	16	—	—	+	—	—
46	2	—	+	+	—	—
47	2	—	—	+	—	—
48	4	—	—	—	+	—
49	25	—	—	—	+	—
50	19	—	+	+	—	—
51	19	+	—	—	—	—

[1] The figures in this column show the strain number of the new strains isolated following a second period of irradiation.

[2] This column shows the strain number of the organisms selected for a second period of irradiation. The requirements of the strains can be seen from Table 3.

TABLE 3.

The individual requirements of the strains following a single and a double period of irradiation.

Single irradiation		Double irradiation			
Primary requirements		Primary requirements		Secondary requirements	
adenine	(2)	adenine	(2)	arginine	(47)
adenine + *thiamine*	(4 and 28)	adenine	(2)	arginine + uracil	(46)
		adenine + thiamine	(4)	leucine	(48)
adenine + *histidine*	(24)	methionine	(16)	arginine + uracil	(40)
		methionine	(16)	adenine	(41)
tryptophane	(19 and 29)	methionine	(16)	*thiamine*	(43)
tryptophane + *phenylalanine*	(20)	methionine	(16)	*histidine*	(44)
		arginine	(25)	tryptophane	(47)
arginine	(25 and 36)	tryptophane	(19)	thiamine	(51)
arginine + *uracil*	(14, 31 and 33)	tryptophane	(19)	arginine + uracil	(50)
methionine	(16)				
leucine	(3) (22 and 39)				

The numbers in brackets are strain numbers of the organisms requiring the substances mentioned.

In Table 3 the first column shows the nine different types of deficiencies encountered after a single period of irradiation. The second column shows the requirements of the five strains selected for a further period of irradiation and the third column shows the additional requirements of the strains after the second period of irradiation.

Two new deficiencies were isolated following the second irradiation. Thus out of a total of 13 irradiations 11 different deficiencies were found. In Table 3 these 11 deficiencies are in italics.

Several of the deficiencies occurred more than once. The arginine + uracil deficiency was isolated on six separate occasions in strains 14, 31, 33, 40, 46 and 50 and was the most frequent, the leucine deficiency occurred three times in strains 22, 39 and 48, and the tryptophane deficiency in Nos. 19, 32 and 49. Strains 25 and 47 required arginine, 2 and 41 required adenine, 4 and 28 required adenine + thiamine, and 43 and 51 required thiamine. Thus all these deficiencies occurred twice each, and the remaining four deficiencies occurred only once.

DISCUSSION.

Working with *E. coli*, Roepke, Libby and Small isolated six deficient strains, with four different types, from 1,994 unirradiated cells and 42 deficient strains, with only six different types, from 6,147 irradiated cells. Roepke and Mercer isolated a further 29 strains from 4,420 irradiated cells. Tatum (1945) isolated

16 deficient strains of *E. coli* from 1,741 isolations after irradiation and one deficient strain from 1,902 isolations with unirradiated material. In the present work with *Ps. aeruginosa* the ratio of the number of deficient cells to the number of irradiated survivors was very small in comparison to the numbers reported by these workers and no deficient strains were detected among unirradiated cells. Apparently the treatment with X-rays was responsible for the production of the deficient strains.

Methods 1, 2, and 3 yielded no deficient strains although some cultures with a delayed growth in the basal medium were isolated. Davis (1950) reported the isolation of strains of *E. coli* with delayed growth following irradiation and he devised the penicillin technique to facilitate the isolation of nutritionally deficient strains. Thirty thousand colonies were obtained and tested by Methods 1 and 2. These colonies were from direct survivors of the radiation treatment. A further thirty thousand were obtained and tested by Method 3 and thus had a preliminary period of incubation in an enriched broth before testing. Only 3,000 survivors from the radiation were involved in these thirty thousand cells. Thus Methods 1, 2, and 3 were ineffective because of the low number of survivors tested. Treatment of sixty thousand cells by Method 4 omitting the period of incubation in enriched broth yielded no deficient strains so that apparently this incubation was necessary for the appearance of the deficient strains. With the preliminary period of incubation Method 4 was particularly valuable as it allowed the examination of 100 times the number of cells examined by Method 3 and one to four deficient strains were isolated per twenty thousand survivors.

The use of solutions containing several growth substances for the identification of the individual requirements of the strains proved very useful, firstly for a rapid screening of the strains, and secondly for the identification of the requirements of strains requiring more than one factor. If the two factors were in different mixtures stimulation was shown by a line of growth midway between the two cylinders. If the two factors were in one mixture they were detected when the solutions in the mixture were tested separately on the one plate.

SUMMARY.

Nineteen different nutritionally deficient strains of *Pseudomonas aeruginosa* were isolated. Nine were isolated following a single period of irradiation and the remaining ten were obtained by irradiating five of the first nine strains for a second time. Eleven different deficiencies were found, namely for adenine, adenine+thiamine, adenine+histidine, methionine, tryptophane, tryptophane+phenylalanine, leucine, arginine, arginine + uracil, thiamine or histidine. Four of these deficiencies required two substances and were isolated following a single period of irradiation. The arginine + uracil deficiency was noted most frequently (in six of 25 mutants).

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DESCRIPTION OF *CLOSTRIDIUM BOTULINUM* TYPE D RECOVERED FROM SOIL IN SOUTH AUSTRALIA

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In 1922 Bull recorded the presence of *Cl. botulinum* in forage which was implicated in the occurrence of forage poisoning, i.e. botulism, in horses in South Australia. Further investigations were carried out on outbreaks of the disease as they occurred, and study was made of the toxic cultures obtained. Throughout these investigations the toxin of only Type C of the species *Cl. botulinum* was demonstrated (Bull, unpublished). In 1924 he investigated an outbreak of the disease in horses on a property near Swan Reach and the evidence again incriminated toxin of *Cl. botulinum* Type C in fodder as the cause of the disease. He examined samples of soil from the property and obtained from a sample of surface soil a culture containing toxin, which, after ingestion by guinea-pigs, produced death from botulism but was not neutralized by antisera against Types A, B and C. He concluded that *Cl. botulinum* of an undetermined type was present in the soil sample, but that it had no epidemiological relationship to the outbreak of botulism in horses. In 1935 he obtained from Dr. E. M. Robinson of the Veterinary Research Station, Onderstepoort, a sample of antiserum prepared against the toxin of a South African strain of *Cl. botulinum* Type D. This protected experimental animals against the toxin of the culture from the surface soil, thus indicating that the culture contained *Cl. botulinum* Type D. It may be recalled that in Western Australia, too, although the existence of *Cl. botulinum* Type D has subsequently been recorded by Bennetts and Hall (1938), who found evidence of its presence once in hay and once in chaff, Type C occurs much more frequently, and that it is the type usually responsible there for botulism in sheep due to the consumption of rabbit carrion.

Frequent unsuccessful attempts to obtain the organism in pure culture were made by Bull, who believes the failure was due to two main causes, viz. the presence of a sporulating "spreader" and the tendency of the strain of *Cl. botulinum* to give rise to atoxinogenic mutants in culture media. Numerous apparently pure cultures were obtained which, although they had the morphological and colonial appearance of *Cl. botulinum*, failed to produce toxin in cultures. In view of subsequent improvements in anaerobic techniques and the development of methods of suppressing swarming by clostridia on solid media, it was considered worth while to attempt again to obtain a pure culture of *Cl. botuli-*

num from the old toxic culture obtained from surface soil in South Australia. This was found possible, but it is of interest and significance to note that, with one exception, all the isolated colonies we have obtained from the culture have proved to be atoxic.

Since *Cl. botulinum* Type D has apparently not hitherto been isolated in pure culture from Australian sources, the characteristics of this strain, referred to as "P.S.a," are here given in some detail.

Methods.

The method used for the isolation was based on the observation of van Goidsenhoven and Bertrand (1935) that they were able to control and inhibit the spreading tendency of *Cl. septicum* by incorporating "Sodium Luminal" (sodium phenobarbitone) in the culture medium; the addition of 0·5 p.c. to the blood agar gave a medium on which discrete colonies of *Cl. septicum* were obtained instead of the usual continuous swarm-film. We applied this technique to the *Cl. botulinum* material, which contained a plectridial swarmer as well as clostridial forms; by sowing from a 24-hour V.F. liver piece broth culture on to blood agar containing 0·1 p.c. of "Sodium Luminal" and then incubating the plates anaerobically by the method of Fortner (1928), we obtained eventually discrete well-separated colonies, cultures from one of which were highly toxic.

Description of the pure strain.

Morphology. Straight, round-ended, Gram-positive rods occur singly, in pairs and in short chains (Fig. 1). In a smear made from a 24-hour culture in V.F. liver piece broth the rods have a length of from 3·0 to 8·5μ and a width of 0·9 to 2·0μ. Spores are seen after 24 hours in cultures in Hall's cooked brain medium; they are oval and sub-terminal, and swell the rods slightly (Fig. 2).

Surface colonies. When first isolated, this strain, like the plectridial contaminant which originally was associated with it, swarmed readily over the surface, but after isolation and storing in pure culture over a period of months it readily formed discrete colonies on blood agar plated even without "Sodium Luminal." These colonies are irregular, non-hemolytic, with blunt processes and some very fine out-growths with thicker nodes at intervals. (Fig. 3.)

Fermentations. These tests were made in the fermentation base described by Spray (1936) with 1 p.c. test substance separately sterilized by filtration, and enriched with 10 p.c. sterile inactivated ox serum. Acid reaction and growth stimulation were produced with glucose, fructose, galactose, maltose, dextrin and inositol, but not with arabinose, xylose, rhamnose, sucrose, lactose, trehalose, raffinose, inulin, glycogen, glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol or amygdalin.

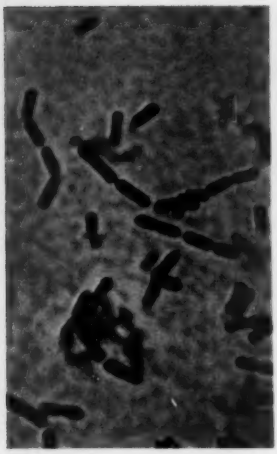

Fig. 1.

Fig. 2.

Fig. 3.

Cl. botulinum Type "P.S.a."

Fig. 1. Smear from 24-hour culture in liver piece broth. Gram stain. × 900.

Fig. 2. Smear from 24-hour culture in Hall's cooked brain medium. Gram stain × 900.

Fig. 3. Surface colony on 10 p.c. sheep blood VF agar. (Fortner method). × 10.

Indole: slight reaction. Nitrate: not reduced. Spray's Iron-Milk: clotted and peptonized after 4 days. Spray's Iron-Gelatin: liquefied in 48 hours at 37° C. Hydrogen sulphide: demonstrated by blackening of cooked brain medium with iron wire (Hall and Petersen, 1924) and of bismuth carbonate agar (Pacheco and Péres, 1940) within 48 hours. Cooked meat medium: meat particles not reddened or digested. Coagulated serum: not liquefied.

Toxicity and neutralization tests. A Seitz E.K. filtrate when tested on pairs of mice and guinea-pigs gave the following results: subcutaneous m.l.d. for mice was $0{\cdot}25 \times 10^{-4}$ ml. and for guinea-pigs was $0{\cdot}5 \times 10^{-4}$ ml. Mice died when fed with biscuit soaked with 0·5 ml. of filtrate. Neutralization tests with anti-botulinum sera Types A, B, C and D resulted in protection by anti-D only. We have also prepared an antiserum against our own strain of which 1 ml. neutralizes between 250,000 and 500,000 m.l.d. of toxin. The anti-"P.S.a" serum, in a dose of 0·025 ml. failed to neutralize test doses containing 8 m.l.d. of Type A, 10 m.l.d. of Type B or 80 m.l.d. of Type C toxin.

Comparison with South African strain. In order to confirm the determination of the "P.S.a" strain as belonging to Type D it was compared with the "classical" South African *Cl. botulinum* Type D. Through the kindness of Dr. M. Sterne, a culture labelled "D6e" and a sample of antiserum were re-

ceived from Onderstepoort. The cultural reactions were compared and a close agreement observed; the "D6e" gave the same fermentation reactions as the "P.S.a" strain. Our findings differ in two particulars from those reported by Meyer and Gunnison (1928, 1929) in U.S.A. with South African strains, by Robinson (1930) and by Mason and Robinson (1935) in South Africa, and by Prévot and Brygoo (1950) in France with a French strain. In spite of repeated attempts we have never observed evidence of fermentation of sucrose, lactose, glycerol or dulcitol by "P.S.a" or by "D6e" either in Spray's fermentation base enriched with 10 p.c. inactivated horse serum or in a Liebig's meat extract peptone base as used by Meyer and Gunnison. Cross-neutralization tests with anti-"P.S.a" and anti-"D6e" sera gave the following results: 1 ml. anti-"D6e" serum neutralized 200 m.l.d. "P.S.a" toxin and 4,000 m.l.d. "D6e" toxin; 1 ml. anti-"P.S.a" serum neutralized 4,000 m.l.d. "D6e" toxin and 20,000 m.l.d. "P.S.a" toxin. This may be restated as follows: the particular sample of "P.S.a" antiserum was 5 times as effective against "P.S.a" toxin as against "D6e" toxin, whereas "D6e" antiserum was 20 times as effective against "D6e" toxin as against "P.S.a" toxin. It would appear therefore, that, although the "P.S.a" strain belongs to Type D of *Cl. botulinum*, both it and the "D6e" strain contain more than one toxic fraction, but in different ratios. This is probably explicable in terms of the finding by Mason and Robinson (1935) that Type D toxin contains not only a large quantity of a specific D fraction but a small quantity of C fraction, and that the C fraction itself may consist of two subfractions C_1 and C_2.

Atoxinogenic mutation It is believed that the culture is pure since surface colonies on blood agar are invariably of the one type. Isolates are similar in morphology and in most cultural characters, but differ strongly in capacity to produce toxin, and, incidentally, in fermentative ability; a small proportion produce a potent toxin, but the rest produce none detectable in 0·5 ml., thus indicating a high mutation rate towards absence of capacity to produce toxin. This is illustrated by the following experiment.

A culture in cooked brain medium of the pure "P.S.a" strain which had been stored in a sealed ampoule for 11 years was sown into liver piece broth; the resulting culture was streaked on the surface of blood agar and incubated anaerobically. Discrete and apparently identical colonies readily grew without the aid of "Sodium Luminal" provided the plates were suitably dried beforehand. Twelve such colonies were picked into liver piece broth. Tests on mice showed that only one of the single colony isolates (Isolate 9), produced toxin. Isolate 9, like the mother culture, clotted and completely peptonized Spray's iron-milk medium in 4 days, whereas the atoxic isolates required 6–10 days for complete peptonization. Isolate 9, like the mother culture, produced acid from glucose, levulose, maltose and dextrin, whereas the 11 atoxic isolates produced no change with any of them.

This experiment suggests that toxinogenicity was linked with capacity to ferment glucose, levulose, maltose and dextrin in the test medium and with a somewhat stronger proteolytic activity as judged by peptonization of milk.

DISCUSSION.

The evidence presented suggests that the "P.S.a" strain of *Cl. botulinum* belongs to Type D, that it is inherently unstable at least under our cultural conditions, and that it yields a high proportion of spontaneous mutants which lack the capacity to produce toxin in culture media and, incidentally, usually are deficient in fermentative ability.

It is suggested that atoxic mutation is the main reason why difficulty was experienced in "purifying" the original culture, since the chance of selecting toxinogenic colonies would appear to be small. That this tendency is not confined to the particular strain studied by us, nor even to the particular toxinogenic type, is evident from the literature; indeed, it has been suggested that many instances of alleged contamination of presumed pure cultures of *Cl. botulinum* by *Cl. sporogenes* are explicable in terms of atoxinogenic mutation (Hadley, 1927). Thus Gunnison and Meyer (1929) found from 6 to 85 p.c. of non-toxic isolates from toxic cultures of various Type A strains; Schoenholz and Meyer (1925) worked with several Type B strains that, after having lost their toxin-producing capacity, still gave their original specific agglutination reactions; Bengtson (1922) reported that Type C gave rise to non-toxic variants; Meyer and Gunnison (1928) in a study of Type D strains reported that "toxic and non-toxic variants may be isolated from fluid cultures" and Robinson (1930), referring to Types C and D, stated that "in all the types the writer has collected, non-toxic colonies have been encountered, and often they are frequent."

Some comment is necessary upon the fermentation reactions. It is to be noted that in our hands fewer substances were fermented by both the Australian and South African strain than have been reported abroad. This virtually disposes of the possibility that our strains were contaminated, since this would almost certainly result in an increase in the range of fermentable substances. In our tests the base was that described by Spray, the carbon sources were added as separately sterilized solutions and it was found necessary to enrich with sterile inactivated horse serum. Under these conditions fermentation was recognized by greatly stimulated growth with little or no gas formation but a large fall in pH as judged by spot tests with methyl red as external indicator; and with both the South African strain and the Australian strain virtually the same reactions were obtained. They differ from the published descriptions in that lactose, sucrose, glycerol and dulcitol were not fermented. To examine the possibility that these discrepancies were related to the use of Spray's fermentation base the tests were repeated with Meyer and Gunnison's test base (using Difco Bactopeptone). The only difference observed was that "D6e," "P.S.a" and the toxic "Isolate 9" all produced acid with dextrin in Spray's base *plus* horse serum but not in Meyer and Gunnison's base. It is conceivable that acid

production with dextrin in Spray's base containing inactivated horse serum might have been the result of dextrinolytic enzyme surviving inactivation, but the failure of "P.S.a" and particularly "D6e" to produce acid with lactose, sucrose, glycerol and dulcitol either in Spray's base or in Meyer and Gunnison's base remains unexplained. One possibility is that our findings may be related to our practice of avoiding heat sterilization and possible hydrolysis of test substances. Another is that our culture media lack growth factors or coenzymes essential for utilization of some test substances. The most likely possibility is a true loss of fermentative capacity during subculturing and conservation in our laboratory. This is rendered more plausible by our finding that, when tested recently after a further two years' sojourn in cooked brain medium, the "P.S.a" strain but not the "D6e" strain was no longer able to produce acid from galactose in either of the two fermentation bases. Our experience emphasizes the view that, although fermentation reactions are a useful and valuable aid in the determination of Clostridia, the possibility of mutational loss must be always considered.

SUMMARY.

For the first time a pure strain of *Cl. botulinum* Type D has been isolated from an Australian source; this derived ultimately from soil in South Australia. Morphological and cultural characters are described. Toxicity and neutralization tests and comparison with a classical strain have confirmed the identity of the organism. The rarity of toxinogenic isolates from apparently pure cultures of the Australian strain suggests a high mutation rate towards loss of toxinogenicity.

Acknowledgments. Thanks are due to Dr. L. B. Bull for providing the original cultures and for advice and assistance in preparing the paper.

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STUDIES ON THE COMPLEMENT FIXING ANTIGEN OF INFLUENZA VIRUS

I. PURIFICATION OF ANTIGEN

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From many points of view, one of the most important phenomena associated with infection by influenza viruses is the production of a "soluble" antigen which when mixed with specific convalescent serum will fix complement. On the practical side, this reaction provides the most sensitive and reliable test for recent infection. It has the special virtue that an influenza A antigen reacts with all anti-A convalescent sera irrespective of the immunological subtypes of virus concerned, similarly a B antigen reacts with all anti-B sera. There is no cross-over between the two types. All the evidence points to the likelihood that these species specific antibodies are produced in response to the stimulus of the soluble C.F. antigen. This antigen is quite distinct from the infective virus particles and it is basic to an understanding of virus reproduction in the cell to determine if possible the chemical nature and source of the specificity of the C.F. antigen. Several possibilities exist but until some acceptable picture of the process of intracellular virus replication is available, it is almost meaningless to present alternatives such as virus precursor, virus breakdown product or by-product of a cellular metabolism altered by virus action. It may be noted, however, that Hoyle (1950) has recently put forward a hypothesis which at least allows the possibility of an experimental approach. He has claimed that the "virus consists of an aggregate of soluble antigen and specific antigen enclosed in a lipoidal envelope. The soluble antigen is probably the fundamental nucleoprotein of the influenza virus."

Any investigation by chemical techniques of the relationship of the soluble antigen to the virus particle and to the host cell necessarily involves as a preliminary step the purification of the soluble antigen. No attempt has been made by previous workers to obtain highly active preparations of the latter and apart from the fact that it is sedimentable in the ultracentrifuge, very little is on record in regard to its physical or chemical qualities.

In preliminary work the technique described by Donnelley (1951) of extracting antigen from infected mouse lungs was used to obtain starting material for chemical work. It was found, however, that extracts of lungs from 18-day-old

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embryo chickens inoculated in the amniotic cavity with virus 48 hours previously had titres often 10-fold higher and in the experiments reported such preparations have served as sources of the antigen.

Materials and Methods.

Virus Strain. The classical influenza strain MEL (type A) was cultivated in the allantoic cavity of 11-day-old hen eggs and the allantoic fluid harvested after 42 hours.

Buffered Saline. These solutions consisted of 9 parts of saline (0·9 gm. NaCl per 100 ml.) mixed with 1 part of M/15 phosphate buffer of pH 7·0 or 7·4 (Clark, 1920).

Buffers for Solubility Tests. The following are the buffers used for solubility tests:

pH 3	0·04 M KCl;	0·05 M glycine;	0·01 M HCl
pH 4	0·04 M KCl;	0·062 M acetic acid;	0·01 M KOH
pH 5	0·04 M KCl;	0·015 M acetic acid;	0·01 M KOH
pH 6	0·04 M KCl;	0·011 M acetic acid;	0·01 M KOH
pH 7	0·04 M KCl;	0·012 M cacodylic acid;	0·01 M KOH
pH 8	0·04 M KCl;	0·013 M veronal;	0·01 M KOH
pH 9	0·04 M KCl;	0·010 M veronal;	0·01 M KOH
pH 10	0·04 M KCl;	0·015 M glycine;	0·01 M KOH
pH 11	0·04 M KCl;	0·010 M glycine;	0·01 M KOH

The ionic strength (μ) of all buffers was 0·05.

Complement Fixation Technique. The method used was the micro-technique described previously by one of us (Donnelley, 1951). Briefly, two drops (0·04 ml.) of the antigen solution are diluted serially in twofold steps into tubes containing one drop of calcium magnesium saline and one drop of 3 $H.D_{50}$ of complement. One drop of optimally diluted mouse anti-serum is added and the tubes then incubated for 60 minutes at 37° C. One drop of 3 p.c. sensitized sheep cells is added and the tubes incubated for a further 30 minutes at 37° C. The dilution of antigen showing 50 p.c. haemolysis is taken as the end point.

Definition of Unit. A solution is said to have a titre of x units when a dilution of $1/x$ in 0·04 ml. volume used in the C.F. technique described (total volume in the test being 0·08 ml.) gives 50 p.c. haemolysis.

In experiments where comparison of results over a period of time was necessary, control samples consisting of purified antigen dried from the frozen state were included in each series of titrations.

Ultracentrifugation. The ultracentrifuge used in the present investigation was designed and assembled by Mr. H. F. Holden of this Institute. The duralumin rotor which is driven by compressed air and runs *in vacuo* holds twelve tubes, each of 8 ml. capacity. The angle of tubes from the vertical is 10°, the maximum and minimum distances from the centre of rotation being 5·9 cm. and 4·5 cm. respectively. The R.C.F. values given in the text are calculated from the mid point of the tubes. The temperature of the rotor (previously cooled to 0° C.) rose to approximately 10° C. during a 3-hour run. The rotor accelerated to 700 r.p.s. in 20 minutes and steady deceleration was maintained to bring the rotor to rest over 30–40 minutes.

Details of the instrument will be published elsewhere at a later date.

Electrophoresis. Electrophoresis was carried out in a Tiselius apparatus equipped with the Philpot-Svennson scanning system (Holden, Ada and Pye, 1952). Analytical runs were done in a 2 ml. fused Pyrex cell. Separation runs were carried out in a 10 ml. two-piece cell. Buffers used were as follows:

Phosphate buffer: $\mu = 0{\cdot}2$ pH 7·0 0·091 M KH_2PO_4 0·055 M KOH
Phosphate buffer: $\mu = 0{\cdot}1$ pH 7·0 0·045 M KH_2PO_4 0·027 M KOH
Veronal buffer: $\mu = 0{\cdot}10$ pH 8·6 0·12 M veronal 0·10 M NaOH
Glycine buffer: $\mu = 0{\cdot}20$ pH 10·0 0·30 M glycine 0·20 M KOH

EXPERIMENTAL.

PREPARATION OF EXTRACT OF INFECTED LUNGS.

Harvest of lungs. 16-day-old chick embryos were inoculated in the amniotic cavity with 0·1 ml. of a 1:10,000 dilution of a freshly harvested allantoic fluid preparation of MEL (A) virus. After 48 hours' incubation at 35° C., the embryos were dissected and the lungs removed. After washing with buffered saline (pH 7·0) to remove excess blood, the lungs were rapidly wiped with blotting paper and ground in a mortar with sterile alundum. The ground tissue was suspended in buffered saline (pH 7·0), using 1 ml. per pair of lungs from each embryo.

Removal of virus. Suspensions of infected lungs contain considerable amounts of virus which can be removed by adsorption to red blood cells. To extracts were added 10 p.c. (V/V) of fresh, washed and packed human red blood cells, the mixture allowed to stand at 4° C for 30 minutes and then centrifuged to sediment the virus-cell complex. This treatment was repeated to remove traces of virus. The soluble antigen remained in the supernatant.

Titre of extract. The average titre of randomly chosen lungs (each extracted with 1 ml. buffered saline pH 7·0) was 430, varying between 160 and 960. The titre could not be correlated with the external appearance of the organ, thus eliminating simple preselection of high titre lungs. Variation in the titre of batches was less marked (240–650), the average value being 480.

FRACTIONATION OF EXTRACT.

Lennette and Horsfall (1940) were the first to obtain separation of the virus particle and the soluble antigen by physical methods. They found that the virus could be removed from infected mouse lung extracts under gravitational forces which left the bulk of the antigen in the supernatant. Filtration studies confirmed the disparity in size of the two particles. On these bases, the soluble antigen was considered to have a molecular weight of between 18×10^4 and 100×10^4. Henle and Wiener (1944) reported the presence of three antigenic components in infected allantoic fluids as measured by complement fixation. One, directly linked with virus infectivity, had a sedimentation constant (S) of 600; a second component, sedimentable at 90,000 *g* (30 S) and possibly identical with the antigen observed by Lennette and Horsfall; and a third non-sedimentable at 90,000 *g* (<30 S). The last two were not associated with infectivity.

In view of these findings, ultracentrifugation seemed to offer a promising means of purifying the soluble antigen. At the time when the present investigation was begun, however, such facilities were not available and an attempt was made using other techniques.

Solvent fractionation.

As Lennette and Horsfall (1940) had previously found that the antigen was stable to treatment with a mixture of ether and alcohol in the cold, fractionation by this technique was undertaken. All procedures were carried out at 0° C.

To the extract was added with stirring over a period of 15 minutes an equal quantity of 70 p.c. (V/V) aqueous methanol, previously cooled to —40° C. After standing for 16 hours to facilitate particulation, the precipitate was removed by centrifugation (2,000 *g* for 15 minutes). The clear red supernatant was decanted and the sediment taken up in one-half of the original volume of buffered saline (pH 7·0). An average of 75 p.c. of the activity was recovered in the sediment, the residual activity remaining in the supernatant. Extension of the time of standing before centrifugation did not increase the amount precipitated. Batch variation made impracticable the elaboration of a more detailed scheme.

Attempts were made to determine whether the antigen was only a minor fraction adsorbed on to a major component or represented a considerable proportion of the sediment. Extraction of the precipitate with buffers of varying pH indicated a minimum solubility at pH 5 rising to a maximum at pH 8. At no value did the activity go into the solution independently of a corresponding amount of the sediment. Washing the precipitate with a large volume of 30 p.c. methanol failed to extract appreciable quantities of the antigen. If the sediment was stirred with a similar solution containing 2·5 p.c. (W/V) glycine, amounts of antigen varying from 25–90 p.c. remained in the supernatant after centrifugation. In each case, however, this was largely paralleled by solution of the bulk precipitate.

The effect of immiscible solvents was next explored. Brief shaking of the solution with ether, either at room temperature or —25° C. (McFarlane, 1942), caused an immediate precipitation of protein and an almost complete loss of activity. A second treatment removed the remainder.

Hoyle has used chloroform treatment of an extract of infected mouse lungs in the preparation of a standard antigen solution. Shaking the concentrate with equal parts of chloroform for half an hour or longer at 0° C. caused the formation of a heavy gel. After centrifugation, the water clear supernatant was found to contain about one-tenth of the activity. None could be recovered from the protein-chloroform gel by treatment with ethanol. If, however, after 10–12 minutes of agitation by hand the mixture was centrifuged (2,000 *g* for 30 minutes), the slightly coloured and opaque supernatant was found to contain 70–90 p.c. of the activity. A second brief treatment with chloroform usually resulted in a loss of activity.

The recent successful application (Morton, 1950) of butyl alcohol to the separation of soluble enzymes from insoluble cell constituents prompted similar experiments. The results in this case were slightly inferior to those obtained

with chloroform. Prolonged exposure to butanol resulted in almost complete loss of activity.

Treatment with chloroform offered the most promise. Highest yields were obtained if the pH was held at 7·4. This was done by resuspending the original methanol precipitate in saline buffered with phosphate at pH 7·4. The chloroform treated solution was frozen and thawed several times, thus removing without loss of titre some denatured material. After concentration by evaporation *in vacuo* from the frozen state, the solution was submitted to electrophoresis in a phosphate buffer (pH 7·0, $\mu = 0{\cdot}2$). Four main components, all migrating toward the anode, were found (Fig. 1). By sampling in the separation cell, the activity was found to be associated with the major peak.

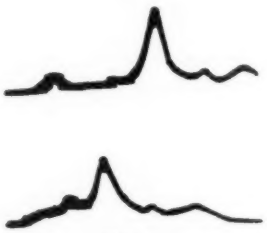

Fig. 1.

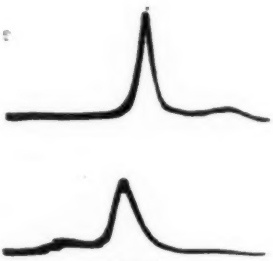

Fig. 2.

LEGEND TO FIGURES.

Electrophoretic behaviour of the complement fixing antigen.

In each Figure, the top and bottom patterns are those obtained in the ascending and descending limbs respectively. In the top pattern, migration is to the left and in the bottom pattern, to the right.

Fig. 1. Pattern obtained after treatment of extract with chloroform. In phosphate buffer (pH 7·0, $\mu = 0{\cdot}2$); field strength = 3·6 volts/cm.; time = 6,000 seconds.

Fig. 2. Pattern given by antigen after complete solvent fractionation. Phosphate buffer (pH 7·0, $\mu = 0{\cdot}2$); 4·5 volts/cm.; 4,320 seconds.

As it appeared that the active component might now represent a major proportion of the protein present, further fractionation with methanol was undertaken. Again batch variation caused inconsistent results and the only practicable solution was to pool several batches and carry out pilot experiments on a small amount.

To the chloroform treated solution was added 60 p.c. (V/V) aqueous methanol (previously cooled to —40° C.) with continual stirring to give a final concentration of 13–17 p.c. (V/V) methanol. The concentration chosen was that which, after removal of the resultant precipitate by centrifugation (2,000 *g*

for 15 minutes), yielded a transparent supernatant. To this supernatant methanol was further added to give a final concentration of 30 p.c. (V/V). After standing for 1 hour, the precipitate was removed by centrifugation. Seventy per cent. of the activity was recovered in the second precipitate which dissolved to give a colourless, slightly opaque solution.

Electrophoresis of the solution in phosphate buffer (pH 7·0, ionic strength 0·2) showed a single, slightly asymmetric peak (Fig. 2).

Fractionation by ultracentrifugation.

At this stage, an ultracentrifuge equipped with a quantity rotor came into operation in the Institute and the possibility that this technique would afford a simpler method of purification was explored.

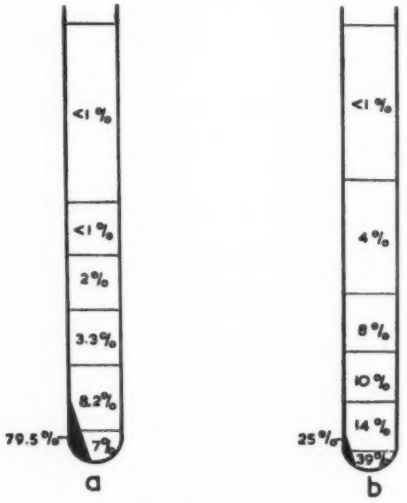

Fig. 3. Illustrating the sedimentation behaviour in sucrose gradients of the complement fixing antigen present in clarified extracts of infected embryo chick lungs.

a. Distribution of activity in a tube after centrifugation at 96,000 *g* for 2 hours.

b. Distribution of activity obtained when top four fractions in diagram (a) are recentrifuged at 96,000 *g* for 2 hours.

A preliminary clarification of the crude extract after removal of virus at 20,000 *g* for 20 minutes caused the sedimentation of particulate material and approximately 5 p.c. of the activity. Resuspension of this sediment (by Pasteur pipette) into buffered saline (pH 7·0) followed by recentrifugation under similar conditions resulted in half the activity remaining in the supernatant. However, if the original solution was layered over concentrated sucrose solution (6 ml. antigen solution over 2 ml. 30 p.c. (W/V) sucrose), only 1 p.c. of the activity was sedimented with particulate material. It seems likely that this small amount of activity was due to incompletely homogenized material.

The pigmented slightly opaque supernatant containing the bulk of the antigen was centrifuged at 98,000 *g* for 2 hours. In order to minimize convection during centrifugation, the solution was introduced into the tubes in layers of increasing density (Pickels, 1943). Sucrose was added to aliquots to give final concentrations varying from 0-12 p.c. (W/V). At the finish of the run the tubes were carefully removed from the rotor, orientated to the upright

position and successive layers removed with the aid of a Pasteur pipette whose point was ground at a slope of 45°. Determination of sucrose concentrations in the fractions obtained in this fashion showed that little convection had occurred during the whole procedure and indicated the reliability of the method of sampling. The distribution of activity found is shown in Fig. 3, a.

It was of interest to determine whether the activity of fractions higher in the tube was due to smaller molecular weight compounds than the bulk of the antigen or to a small portion of the antigen which was loosely packed on the side of the tube and "spilled" into the supernatant when the latter was removed. The top four layers were pooled, largely freed of sucrose by dialysis and recentrifuged under identical conditions as previously. Fig. 3b shows the distribution of activity obtained. Using Pickels' (1943) empirical formula and assuming spherical particles with a density of 1·2–1·3, a mean molecular weight of $1{\cdot}5 \times 10^6$ is obtained. There is no suggestion of compounds of molecular weight less than 5×10^5 having activity.

To purify further the antigen, the sediment and lowest layers were pooled, diluted with buffered saline and centrifuged at 50,000 *g* for 2 hours in a sucrose gradient. The resulting sediment and lowest layer were found to contain 80–90 p.c. of the antigen whilst the bulk of the contaminating smaller molecular weight compounds remained in the higher layers. In order to avoid this second run and the firm packing of the sediments which made resuspension tedious (and of doubtful efficiency), varying amounts of a 30 p.c. (W/V) sucrose solution were layered under the main solution. Under these conditions, the antigen slowly penetrated the concentrated sucrose layer and at the conclusion of the run was only loosely packed at the bottom of the tube. Two and a half ml. of sucrose solution layered under 5·5 ml. of the antigen solution gave most satisfactory results as it was found that smaller molecular weight compounds such as haemoglobin did not penetrate appreciably the high density layer. Generally an almost clear layer of sucrose separated the contaminants from the antigen. Thus the latter could be obtained largely free of smaller sized compounds by removing the solution down to the point where pigmentation due to haemoglobin ceased.

In later experiments purification of the antigen has been carried out in an electrolyte-poor medium with a view to minimizing break-down of the various components during isolation. Infected lungs are ground and extracted with 10 p.c. (W/V) sucrose. The preliminary clarification is carried out under similar conditions as previously. The supernatant is layered over 30 p.c. sucrose and centrifuged at 75,000 *g*. for two hours.

When submitted to electrophoresis in a phosphate buffer (pH 7·0, $\mu = 0{\cdot}2$), this preparation yields a single, slightly asymmetric peak migrating towards the anode (Fig. 4, a).

Comparison of two methods of purification.

As far as has been investigated, both methods yield identical products. Electrophoretically, both preparations behave similarly—at least within the range of variation found between different batches prepared by the one method. In each case a 1 p.c. (W/V) solution of the final product has a titre of between 5,000 and 10,000 units per unit volume. While 40–50 p.c. of the original amount

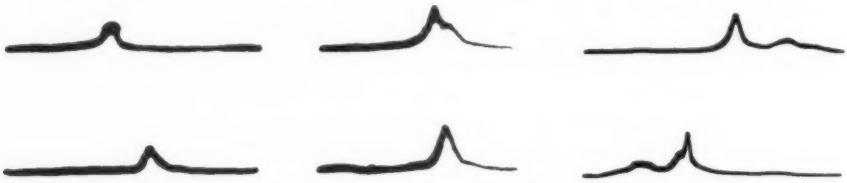

Fig. 4a. Fig. 4b. Fig. 4c.

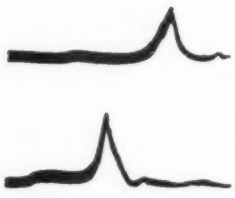

Fig. 4d.

Fig. 4. Behaviour of antigen purified by differential centrifugation.

(a) In phosphate buffer (pH 7·0, $\mu = 0{\cdot}1$); 3·6 volts/cm.; 11,220 seconds.

(b) In veronal buffer (pH 8·6, $\mu = 0{\cdot}1$); 4·7 volts/cm.; 8,040 seconds.

(c) In veronal buffer (pH 8·6, $\mu = 0{\cdot}1$); 4·8 volts/cm.; 4,920 seconds.

(d) In glycine buffer (pH 10·0, $\mu = 0{\cdot}2$); 2·7 volts/cm.; 7,920 seconds.

could be obtained as purified antigen by solvent fractionation, purification by differential centrifugation gave yields of 80 p.c. The latter method was chosen for routine work not only for this reason but also as centrifugation is a more gentle method of treatment than exposure to organic solvents.

PHYSICAL PROPERTIES OF THE ANTIGEN PURIFIED BY ULTRACENTRIFUGATION.

(i) *Solubility and stability.*

Aliquots (0·1 ml.) of a freshly prepared batch of antigen (concentration about 0·2 p.c. (W/V)) were added to 0·4 ml. volumes of buffers whose pH values ranged from 11 to 3. The solutions were stored at 2° C. and samples removed, clarified by centrifugation and titrated at intervals of 1, 2 and 5 days as indicated in Table 1. At the pH values where gross precipitation occurred, the values given in the Table represent those of the supernatants. In these cases solution of the precipitates without loss of activity could be obtained by vigorous resuspension in a pH 7 buffer. As such solutions proved to be unstable, however, this step is not recommended as a means of purification.

TABLE 1.

Solubility and stability of the complement fixing antigen, purified by ultracentrifugation, in buffers of varying pH at 2° C.

Buffer	pH	Titre of solutions kept at 2° C. after 1 day	2 days	5 days
Glycine–KOH	11	530	640	320
Glycine–KOH	10	430	480	480
Veronal–KOH	9	640	480	320
Veronal–KOH	8	640	430	430
Cacodylic acid–KOH	7	530	480	480
Acetic acid–KOH	6	640	530	430
Acetic acid–KOH	5	15*	—	—
Acetic acid–KOH	4	17*	—	—
Glycine–HCl	3	430	240	—

0·1 ml. of 0·5 p.c. (W/V) antigen in 25 p.c. sucrose added in each case to 0·4 ml. buffer.
* Titre of supernatants after removal of gross precipitate by centrifugation.

It is quite clear that the antigen is soluble and reasonably stable at pH values between 6 and 11 but becomes largely insoluble at pH values 5 and 4. This suggests an isoelectric point in the range pH 4–5 which agrees with the value found previously by Bourdillon and Lennette (1940) from electrophoretic data.

It has been found most convenient to keep the antigen in the concentrated sucrose solution in which it is prepared. Storage at —10° C. results in only slow deterioration provided the solutions are not thawed and refrozen frequently. If required to store for long periods, the antigen may be dried from the frozen state *in vacuo* after first removing the bulk of the sucrose by dialysis. Such preparations stored at low temperatures remain active for long periods.

(ii) *Electrophoretic examination.*

Solutions of the purified antigen varying in concentration from 0·3 to 0·8 p.c. (as determined by refractometry) were used.

In phosphate buffer (pH 7·0), a single peak was obtained at ionic strengths (μ) 0·1 and 0·2. Generally the peak was skew, the degree of asymmetry being less marked in the ascending limb.

Four preparations were submitted to electrophoresis in veronal buffer (pH 8·6, $\mu = 0{\cdot}1$) with the following results. A single asymmetric peak was obtained in one case. In another, the ascending boundary showed a tendency to split into two peaks (Fig. 4, b). On both other occasions the descending boundary exhibited a structure similar to the anomaly often observed with some human sera in the same buffer. This took the form of a sharp spike moving with the leading edge of the peak and was associated with a turbid region in the cell (Fig. 4, c).

In one experiment in glycine buffer (pH 10, $\mu = 0{\cdot}2$), a single asymmetric peak was observed in each limb (Fig. 4, d).

DISCUSSION.

The production of large amounts of complement fixing antigen in embryo chick lungs following inoculation with influenza virus has made possible the elaboration of a simple method whereby preparations of high activity may be readily obtained. The high titres can probably be related to the fact that a large proportion of the cells making up the lung at this stage are derived from the respiratory epithelium that buds out from the parabronchii from the 13th day onward to form the respiratory capillaries. As shown by Burnet (1940) these cells are highly susceptible to influenza virus infection, almost complete necrosis being produced with an active virus in 2 days. Similar histological appearances are evident in the lungs harvested for the present work. The embryonic character of the cells, the virtual simultaneity of their infection and the absence of immunological or reparative reaction may all play a part in producing a much higher titre of antigen than is formed in infected mouse lungs.

It is known (work to be reported) that similar preparations from normal embryo chick lungs contain material with fairly similar properties in the ultracentrifuge and electrophoresis apparatus. In view of this, it must be assumed that the preparation obtained by differential centrifugation consists of components (including the complement fixing antigen) from infected cells together with constituents from uninfected cells. At this stage in the purification therefore, it is unlikely that the preparation would behave as a homogenous compound by physical criteria such as electrophoresis. Indeed, there is no reason to expect that the antigen itself is a single molecular species. With this in mind, therefore, the results obtained by electrophoretic examination of the purified preparations are not surprising. The single asymmetric peak obtained in both phosphate and glycine buffers may represent a mixture of particles of reasonably similar size and nett surface charge. A complication present in the patterns obtained in veronal buffer is the feature resembling the β anomaly often found in serum patterns, particularly in this buffer. According to Antweiler and Engelhard (1950), this phenomenon is caused by local electroosmotic disturbances which give rise to anomalous boundaries. The marked dissimilarity in the ascending and descending patterns of some preparations in veronal buffer suggests interaction of the protein with the buffer ion.

The sedimentation behaviour of the preparation suggests that the activity is associated with relatively large particles of approximately microsomal size, confirming the conclusion reached previously by Lennette and Horsfall. A detailed study of preparation in the analytical rotor (unavailable at present) would yield more precise information concerning the size characteristics of the preparation. However, it is felt that further study or attempt at purification by physical methods is unjustified until some indication is obtained by more specific techniques of the composition of the preparation.

SUMMARY.

Lungs of 18-day embryo chicks inoculated in the amniotic cavity 48 hours previously with influenza virus have been found to contain comparatively large amounts of the soluble complement fixing antigen.

Methods of purifying the antigen in extracts of such lungs have been developed involving either fractionation with methanol and chloroform or differential centrifugation in an ultracentrifuge. Both techniques appear to yield similar products, the activities of 1 p.c. (W/V) solutions ranging from 5–10,000 units. However, yields of up to 80 p.c. are obtained by the latter method as compared with 40–50 p.c. given by the former process.

The activity is associated almost completely with particles which fall within the microsomal range. This would correspond to spherical particles of diameter 15 mμ.

An electrophoretic examination of the purified preparation shows a single slightly asymmetric peak in phosphate buffer pH 7·0 and in glycine buffer at pH 10. More complex patterns are given in veronal buffer at pH 8·6.

The purified antigen is rather insoluble at pH 4 and 5 but is soluble and reasonably stable at pH values between 6 and 10. Specimens dried from the frozen state *in vacuo* may be stored for long periods without loss of activity.

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THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE AND GROWTH HORMONE ON THE FAT, WATER AND PROTEIN CONTENT OF MOUSE LIVER

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The ability of crude and partially purified extracts of the anterior pituitary gland to produce a marked infiltration of fat into the liver is a well known phenomenon. Little however is known about the effects of such extracts on the water and protein content of the liver. Two of the anterior pituitary hormones adrenocorticotrophic (ACTH) and growth hormone, have been isolated as electrophoretically homogeneous substances and are known to cause an accumulation of liver fat, but again no detailed studies have been made of their effects on the protein and water content of that organ. The present experiments have been designed to determine the relative effects of crude saline extracts of sheep, ox and hog pituitary glands on the protein, fat and water content of mouse liver and to correlate these results with those obtained after injection of growth hormone and ACTH.

Materials and Methods.

Hormones.

ACTH was prepared from hog pituitary glands by the method of Fishman (1947). Growth hormone was prepared from the anterior lobes of ox pituitary glands according to Wilhelmi, Fishman and Russell (1948). Both hormones were electrophoretically homogeneous.

Crude pituitary extracts.

Anterior lobes of ox and whole sheep and pig pituitary glands were ground with coarse silica in the presence of 0·9 p.c. NaCl and stirred for one hour at 0° C. The extracts were then centrifuged to remove coarse material and the supernatants sterilized by high speed centrifuging for one hour at 34,000 R.C.F. and at 0° C. The supernatant fluid was removed and aliquots withdrawn for the determination of protein N. The volume was then adjusted by the addition of sterile 0·9 p.c. NaCl in such a manner as to bring the concentration of protein N to 2 mg./ml. The extracts were stored at 1° C. in the presence of 0·1 p.c. phenol.

Treatment of animals.

Male albino mice 20 ± 2 gm. body weight were divided into groups, each of 5 mice, and injected subcutaneously with the various preparations. In the case of ACTH and growth

hormone, the dry material was dissolved immediately before use in 0·9 p.c. NaCl and N NaOH added to adjust the solution of pH 8·0. The animals were given liberal supplies of water, but all food was removed immediately following the injections. Seven hours later the animals were weighed, stunned, the cervical vessels severed and the livers removed. After removal of the gall bladder, the livers were dropped into liquid O_2.

Water content.

Each experimental batch of 5 livers was removed from the liquid O_2, placed in a tared flask and rapidly weighed. The flask was then connected to a freeze-drier and evacuated to 10 microns. After 20 hours the flasks were disconnected, reweighed and the water content of the pooled livers calculated. Experimental checks of this method have shown that the results are comparable with those obtained by drying to constant weight at 110° C.

Fat content.

Aliquots of the powdered dried livers were weighed into cellulose thimbles and the fat extracted with acetone in a Soxhlet apparatus.

Nitrogen.

Duplicate samples of the powdered livers were wet-ashed in micro-Kjeldahl flasks with the digestion mixture described by Campbell and Hanna (1937) from which the phosphoric acid had been omitted. The digests were made to a suitable volume and aliquots distilled in the apparatus described by Markham (1942). All distillates were collected in boric acid buffer (Conway, 1942) and titrated with 0·01 N HCl.

RESULTS.

It is clear (Table 1) that the three types of crude extract employed have produced similar alterations to the fat, water and protein contents of the mouse liver. All have produced an increase in liver weight, which was due to a rise in the water and fat contents. It will be noted that whilst there was a decrease in the per cent. water there was an increase in the absolute amount present in the liver, as indicated by the figures expressed in terms of final body weight. The protein content, on the other hand, has decreased both in percentage and in total amount. This finding is not in agreement with that of Campbell (1946) who, working with crude extracts of ox anterior pituitary lobes, stated that the increase in liver weight was due to an increase in all three constituents, i.e. protein, fat and water.

The effect of ACTH is somewhat similar to that produced by the crude extracts since increases in both water and fat contents and a decrease in protein content are apparent. These changes are, to a large extent, proportional to the dose administered and are similar to the results reported by Li *et al.* (1949), although these workers used rats which had been injected with a total of 30 mg. of ACTH over a ten-day period. Li, Simpson and Evans (1949a) used a shorter experimental period. In an 8 hour experiment, using rats and a 5 mg. dose of ACTH, they were unable to show any marked change in the water content of the liver, but this result is perhaps not surprising since only small increases in liver fat were observed.

TABLE 1.

The effect of ACTH, growth hormone and crude pituitary extracts on the constituents of mouse liver.

Five male mice were taken for each group. All animals were starved 7 hours before sacrifice and injections (in all cases 0·5 ml.) were given at zero time. In the case of the crude preparations 1·0 mg. of protein N was injected. The figures quoted are average values, together with standard deviation of average values.

Treatment	Liver wt. gm./100 gm. body wt.	p.c.	Water gm./100 gm. body wt.	p.c.	Fat gm./100 gm. body wt.	p.c.	Protein gm./100 gm. body wt.
			Controls				
Saline	4·33±0·22	69·5	3·01±0·16	6·00	0·26±0·07	23·4	1·01±0·07
			Pituitary extracts				
Hog P.E.*	5·20±0·59	64·8	3·37±0·43	14·5	0·76±0·12	15·6	0·82±0·11
Sheep P.E.*	5·08±0·31	64·9	3·33±0·20	14·9	0·76±0·04	15·5	0·79±0·06
Ox A.P.E.**	5·33±0·20	65·1	3·48±0·12	14·4	0·77±0·09	15·7	0·84±0·13
			ACTH				
0·1 mg.	5·01±0·40	66·7	3·35±0·29	9·5	0·48±0·08	18·5	0·94±0·10
0·5 mg.	5·12±0·30	65·0	3·26±0·25	12·1	0·63±0·13	16·7	0·84±0·09
1·0 mg.	5·32±0·34	65·1	3·47±0·25	12·8	0·68±0·12	16·0	0·85±0·07
			Growth hormone				
0·1 mg.	4·73±0·27	66·0	3·14±0·24	11·4	0·55±0·16	18·1	0·86±0·16
0·5 mg.	4·82 —	66·9	3·21 —	11·1	0·51 —	18·3	0·88 —
1·0 mg.	4·99±0·43	64·9	3·24±0·30	14·4	0·73±0·06	16·7	0·83±0·18

* P.E. = pituitary extract. ** A.P.E. = anterior pituitary extract.

Growth hormone in doses similar to those used in the case of ACTH also produced comparable increases in liver fat. Liver weight also increased, but not to the same extent as occurred when crude extracts, or equivalent amounts of ACTH, were given. This result is apparently largely due to the fact that the water content had not increased to the same extent as occurred in those experiments. Growth hormone is known to cause N retention when injected over a prolonged period (Li, Simpson and Evans, 1949b), but this is not the case in an acute experiment since the protein content of the liver decreased in the present experiments. The present results, as far as the effect on liver fat is concerned, are similar to those reported by Szego and White (1949), who treated mice with 0·2 mg. of growth hormone and starved them for 48 hours. In those experiments, however, these workers found an increase in the protein levels—the explanation of the difference between those results and the present ones probably lies in the difference between the experimental times.

These results are also in agreement with those reported by Weil and Ross (1949), who found that 0·1 mg. of growth hormone injected into mice produced a twofold increase in liver fat in an experimental period of seven hours, but no details were given by these workers on the effects on liver protein. Li, Simpson and Evans (1949a) have also found an increase in liver fat in rats fasted

24 hours and injected with 5 mg. of growth hormone in three divided doses over a six-hour period. No significant effect, however, was observed on the per cent. water and protein.

DISCUSSION.

The present results show that both ACTH and growth hormone are capable of producing marked increases in the fat content of the livers of mice in a short experimental period of seven hours. In so far as these effects have been obtained in the absence of exogenous adrenal steroids they are at variance with the results of Levin and Farber (1950). These workers could find no effect of growth hormone with a dose as high as 5 mg. in a seven-hour experimental period. When, however, cortisone was injected prior to the hormone increases in liver fat were obtained. Since growth hormone has been shown to be active in increasing liver fat in the absence of the adrenals it is difficult to reconcile the action of growth hormone with the need for adrenal steroids.

It is tempting to explain the effects of crude extracts on liver constituents in terms of their assumed contents of growth hormone and ACTH. Whilst it may be assumed that all of the crude extracts employed contained both of these hormones no assays have been carried out and thus the relative concentrations are not known. The effects of the crude extracts of the pituitaries from the three species tested on the liver constituents are similar to the action of ACTH and growth hormone, in so far as the increase in liver fat and the decrease in liver protein is concerned. However, the effect on liver water and total liver weight is such as to preclude correlation with the possible content of ACTH and growth hormone. Thus, with crude extracts of ox anterior pituitary lobes, which are known to contain large amounts of growth hormone and small amounts of ACTH (Reiss and Halkerston, 1950), the increase in liver weight and the effect on the water content is more characteristic of ACTH than of growth hormone. On the other hand, the crude extract of hog pituitary glands, which contain large amounts of ACTH (Burns, Merkin, Sayers and Sayers, 1949) was not as effective in increasing the liver weight and water content as were smaller quantities of the pure hormone. This difficulty in correlating the action of crude extracts with their probable content of growth hormone or ACTH could be due to the *in vivo* effect of one hormone upon the other for it is known that they do, under certain circumstances, exert antagonistic effects (Marx, Simpson, Li and Evans, 1943). In this connection, however, it should be remembered that there is no certainty that growth hormone and ACTH are the only principles active in mobilizing fat to the liver.

SUMMARY.

The effects of crude pituitary extracts, ACTH and growth hormone, on some constituents of the mouse liver have been studied.

Injections of crude saline extracts of anterior lobes of ox and whole hog and sheep pituitary glands led to increases in the liver weight and in the water and fat content. The protein content of the liver was decreased.

ACTH produced an increase in liver weight and in the water and fat content, but the protein content was decreased.

Growth hormone also increased the liver weight, but to a lesser extent than with the other preparations. This was due to an increase in fat, and, to a lesser extent, water. The protein content, as with the other preparations, was decreased.

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MURRAY VALLEY ENCEPHALITIS INFECTION IN THE LABORATORY MOUSE

I. INFLUENCE OF AGE ON SUSCEPTIBILITY TO INFECTION

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During the summer of 1951 there occurred in the Murray Valley in Northern Victoria, an outbreak of severe encephalitis (Anderson, 1952). From the brains of three fatal cases, a virus was isolated on the chorioallantoic membrane of developing chick embryos. This virus has proved to be highly pathogenic for mice which, following intracerebral inoculation, typically develop encephalitic symptoms followed by death (French, 1952). Since it seemed possible that a study of MVE virus in the mouse might provide a useful model for elucidating the behaviour of the virus in man, an investigation of the course of MVE infection in the mouse was undertaken.

In order to approximate to a naturally acquired type of infection, the virus was introduced into mice by a peripheral route. By carrying out infectivity titrations on various tissues removed at different times after inoculation, it was hoped that evidence would be obtained of the way in which the virus spreads within a mammalian host and that it would be possible to discover whether tissues other than those of the nervous system are capable of supporting virus multiplication. However, preliminary experiments showed that in common with many other "neurotropic" viruses, MVE inoculated peripherally caused symptomatic infection far more readily in young than in older animals so that a careful investigation into the age susceptibility of mice to MVE infection became an essential preliminary to the main investigation.

It has frequently been reported that following peripheral inoculation with many of the "neurotropic" viruses, animals may, with increasing age, develop broadly similar types of resistance to symptomatic infection. Working with the viruses of vesicular stomatitis and Western equine encephalitis, Olitsky *et al.* (1936) found that older mice were more resistant than younger mice to infection by peripheral routes, some mice of about 30 days of age showing considerable resistance although others remained susceptible up to the age of 1 year. This resistance was demonstrable using a variety of peripheral routes but no change in susceptibility was shown by mice of different ages inoculated intracerebrally: further, this type of resistance was unrelated to previous exposure to infection or to the presence of antibody. Using the virus of Eastern

[1] This work was carried out while in receipt of a British Colonial Medical Research Studentship.

equine encephalitis, Sabin and Olitsky (1938b) showed that when the intramuscular or intra-abdominal routes were employed, although all 15-day mice succumbed to infection, 40 to 50 p.c. of 1-month-old mice and 95 p.c. of 3-month-old mice were resistant. However, such resistant mice developed fatal encephalitis if inoculated by the intracerebral, intranasal or intrasciatic routes. Lennette and Koprowski (1944) made a thorough survey of the susceptibility of mice to nine different "neurotropic" viruses, inoculated both by peripheral and by intracerebral routes. They concluded that with increasing age, mice inoculated intracerebrally showed no significant change in susceptibility to infection with any of these viruses. However, with age, there developed a resistance against peripheral infection by the following seven of these nine viruses: Bwamba fever, Herpes simplex, St. Louis encephalitis, West Nile, Japanese B encephalitis, Western equine and Eastern equine encephalitis. Of all those viruses tested which proved capable of inducing disease after inoculation by peripheral routes, only Venezuelan equine encephalitis was equally infective to young mice and to old mice following peripheral inoculation.

Exploratory experiments showed that MVE virus resembled the majority of "neurotropes" in infecting young mice more readily than older mice following peripheral inoculation. The development of this resistance with age was investigated by comparing the mortality of mice of graded ages inoculated with several dilutions of virus by both intracerebral and intramuscular routes. Parallel titrations of the same test virus were carried out in chick embryos.

Materials and Methods.

Mice.

All mice used were bred in the Hall Institute. This stock is of mixed-coat colour and although harbouring a strain of TO-type mouse encephalomyelitis virus similar to that described by Theiler (1937), is perfectly healthy. The age of all mice was accurately known.

Virus.

Murray Valley encephalitis (Strain 1), isolated on the chorioallantoic membrane of the developing chick embryo (French, 1952). A single pool of virus was used throughout these experiments and was prepared as follows: 0·03 ml. of a 10^{-1} suspension of 10th mouse passage material was inoculated intracerebrally into healthy 21-day-old mice. On the 5th day after inoculation, the brains were harvested, ground with sterile alundum, and made up in nutrient broth pH 7·6, to a 10 p.c. weight by volume suspension. This material was centrifuged at 2,500 r.p.m. for 10 minutes after which the supernatant was pipetted into ampoules and stored at —70° C.

Preparation of dilutions.

A separate ampoule of the virus pool was used for each experiment. Ampoules were removed from —70° C. as required, thawed rapidly in a 37° C. water-bath, and ten-fold dilutions were prepared in nutrient broth pH 7·6, 0·25 ml. of virus being added to 2·25 ml. of broth. The original 10 p.c. suspension was taken as the 10^{-1} dilution.

Inoculation of mice.

All inoculations were made with a 1 ml. tuberculin syringe, the dose in all cases being 0·03 ml. per mouse. Intracerebral inoculations were made under light ether-chloroform anaes-

thesia, into the left cerebral hemisphere. Intramuscular inoculations were carried out without anaesthesia, into the gastrocnemius muscle. In the case of mice of 15 days of age and older, the inocula probably remained reasonably well localized within the muscle but in the case of younger mice, there is no doubt that inocula of this size are not completely localized.

Inoculation of eggs.

Fertile eggs which had been incubated for 11 days at 39° C., were inoculated on the chorioallantoic membrane by the technique of Beveridge and Burnet (1946), using an inoculum of 0·05 ml. per egg. After inoculation, they were incubated for 40 to 48 hours at 35° C. when the chorioallantoic membranes were removed and examined for evidence of MVE infection.

EXPERIMENTAL.

As mice of the required age became available, groups of at least 5 were inoculated by each route. The same virus pool was used throughout the experiments and in every case, within any one age group, a single set of dilutions was used to inoculate by both routes. Dilutions were kept in an ice-bath while inoculations were carried out. The mice were observed daily over a period of 3 weeks and any symptoms of sickness were noted.

The initial symptom following any route of inoculation was usually twitching of the ears. A little later, symptoms of cerebral damage developed and shortly afterwards, death ensued. An occasional mouse developed paralysis of the hind limbs (see below) but paralysis of the fore limbs has never been observed. The incubation period and duration of the disease varied with age of mice and virus dosage, being shorter in younger than in older mice and also shorter in mice inoculated with low dilutions than with higher dilutions of virus. In no case has recovery been observed: the appearance of symptoms is always followed by death of the affected animal. Thus, throughout these experiments, susceptibility to infection was measured by recording the numbers of mice dying amongst those inoculated with each dilution, the LD_{50} being calculated for both the intracerebral and intramuscular routes by the method of Reed and Muench (1938).

Egg titrations were carried out in parallel with the mouse titrations using the identical set of dilutions inoculated into the mice, 6 eggs being used for each dilution. The membranes were removed from inoculated eggs after about 40 hours' incubation at 35° C. and were examined for the presence of pocks, i.e. for evidence of MVE infection (French, 1952). The number of infected eggs in each group was recorded and ID_{50} values were calculated by the method of Reed and Muench.

Results.

In the main series of experiments, mice ranging from 5 to 28 days of age were inoculated, the results of intracerebral and intramuscular titrations being shown in Table 1 and Fig. 1. In addition a group of old mice (280 days) was included.

TABLE 1.

Age resistance in mice to Murray Valley encephalitis virus.

Dose: 0·03 ml. per mouse by either the intramuscular or intracerebral route.
Numerator = Number of mice died. Denominator = Number of mice inoculated.

Age (Days)	Route of Inoculation	Dilutions of Virus. 10–8	10–7	10–6	10–5	10–4	10–3	10–2	10–1	LD_{50}
5	I.Cer.	0/6	2/6	6/6	6/6	6/6	—	—	—	$10^{-6.7}$
	I.Musc.	0/7	1/6	6/6	6/6	—	—	—	—	$10^{-6.6}$
10	I.Cer.	0/6	2/6	3/6	6/6	6/6	6/6	—	—	$10^{-6.3}$
	I.Musc.	—	0/6	2/6	6/6	6/6	6/6	—	—	$10^{-5.7}$
12	I.Cer.	0/6	5/6	3/7	7/7	—	—	—	—	$10^{-7.0}$
	I.Musc.	0/6	1/6	1/5	6/7	5/5	—	—	—	$10^{-5.7}$
15	I.Cer.	—	0/6	2/6	6/6	6/6	6/6	—	—	$10^{-5.7}$
	I.Musc.	—	0/6	0/6	4/8	7/7	7/7	—	—	$10^{-5.0}$
17	I.Cer.	—	0/6	1/6	6/6	6/6	—	—	—	$10^{-5.6}$
	I.Musc.	—	0/5	0/5	1/5	6/6	—	—	—	$10^{-4.7}$
21	I.Cer.	0/6	0/6	0/6	4/6	6/6	—	—	—	$10^{-5.2}$
	I.Musc.	—	—	0/6	0/6	1/6	5/6	1/6	—	$?10^{-3.0}$
28	I.Cer.	0/5	0/6	0/6	3/6	6/6	6/6	—	—	$10^{-5.0}$
	I.Musc.	—	—	0/6	0/6	1/6	1/6	1/6	0/6	$<10^{-1.0}$
280	I.Cer.	—	0/6	0/6	3/6	4/6	6/6	6/6	—	$10^{-4.7}$
	I.Musc.	—	—	—	—	0/6	0/6	0/6	0/6	—

LD_{50}s calculated by the method of Reed and Muench.

It will be seen that between the ages of 15 and 30 days, there is a sharp increase in resistance to infection by peripheral inoculation although susceptibility to intracerebral infection shows little real change. There are some irregularities in the results of peripheral inoculation at 21 and 28 days, but it is clear that by 28 days, the great majority of the mice inoculated by this route have developed resistance almost as complete as that shown by aged mice.

In contrast to results reported with other viruses, there is also a significant but much less marked increase with age in resistance to intracerebral infection.

On 5 separate occasions the virus pool was titrated by the chorioallantoic method, and, with one ex-

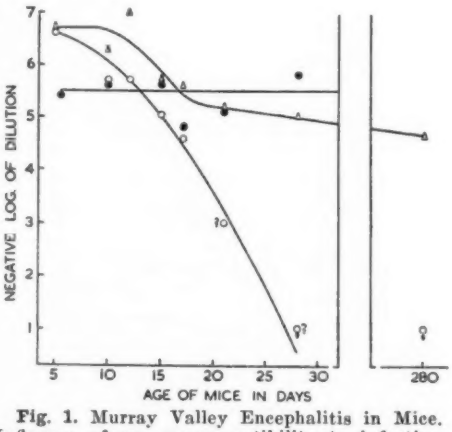

Fig. 1. Murray Valley Encephalitis in Mice. Influence of age on susceptibility to infection by the cerebral and intramuscular routes.

△——△ Intracerebral titre in mice. Dose 0·03 ml. per mouse.

O——O Intramuscular titre in mice. Dose 0·03 ml. per mouse.

•——• Titre in eggs by chorioallantoic route. Dose 0·05 ml. per egg.

ception (4·8) all ID_{50} values lay between 5·4 and 5·8, so that 5·5 may be taken as the median.

Examination of Fig. 1 shows that when an attempt is to be made at isolation of very small amounts of MVE virus, the method of choice is intracerebral inoculation of suckling mice, both egg inoculation and intracerebral inoculation of standard 3 to 4-week-old mice being significantly less sensitive.

A point of interest emerging from these experiments concerns the proportion of mice in which infection is manifested by paralysis of the hind limbs. This symptom is very rarely seen in younger mice but was shown by 26·5 p.c. of mice infected at 14 days or older. A significant proportion of older mice inoculated intracerebrally also show this symptom (Table 2).

TABLE 2.

Murray Valley encephalitis virus in mice.

Symptoms		Age in days (at time of inoculation)			
		5 to 13		14 to 280	
		Route of inoculation		Route of inoculation	
		I.Cer.	I.Musc.	I.Cer.	I.Musc.
Encephalitis	Ratio	79/81	76/77	73/82	25/34
	p.c.	97·5	98·7	89	73·5
Paralysis	Ratio	2/81	1/77	9/82	9/34
	p.c.	2·5	1·3	11	26·5

DISCUSSION.

It has been shown that MVE virus resembles most other "neurotropic" viruses in passing much more readily from the periphery to the central nervous system in young than in older mice. No fully satisfactory explanation of this phenomenon has been provided. It is possible that the changing incidence with age of posterior paralysis following peripheral inoculation, may provide a clue. Up to the age of 13 days, only one mouse of 77 showed this symptom, while 26·5 p.c. of 34 older mice did so. Sabin and Olitsky (1938a) obtained somewhat similar results in mice inoculated intramuscularly with Western equine encephalitis virus. The change from predominantly encephalitic symptoms in 15-day mice, to an incidence of 70 to 80 p.c. of flaccid paralysis in 21-day mice was a striking feature of their experiments. Viruses such as the GD VII strain of mouse encephalomyelitis which are thought to reach the central nervous system from the periphery by axonal spread, almost invariably produce paralysis of the inoculated limb as their initial symptom. There is, therefore, a strong suggestion that in young animals (14 days), MVE virus reaches the central nervous system by some route other than via the axons. In the mice in older age groups developing flaccid paralysis, the virus may have reached the

spinal cord by axonal spread along the peripheral nerves, but the appearance of this symptom in 11 p.c. of such mice inoculated intracerebrally, must be kept in mind. At least it seems clear that between 2 and 4 weeks of age, a significant change takes place in tissue responses to introduction of this virus. Experiments to compare the site and extent of multiplication of MVE virus after intramuscular injection in young and in old mice were therefore undertaken and are reported in the second paper of this series.

SUMMARY.

The susceptibility of mice to infection with MVE virus has been tested at ages from 5 to 28 days. A group of old (280-day) mice was also studied.

Suckling mice are infected intracerebrally with smaller doses of virus than are required for older mice.

After peripheral inoculation, fatal encephalitis ensues much more readily in the younger mice, the main change in susceptibility to infection taking place between 17 and 28 days of age.

Acknowledgments. In the preparation of this paper, the author is much indebted to the help and criticism of Sir Macfarlane Burnet. Miss Mary Cook kindly drew Fig. 1.

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MURRAY VALLEY ENCEPHALITIS INFECTION IN THE LABORATORY MOUSE

II. MULTIPLICATION OF VIRUS INOCULATED INTRAMUSCULARLY

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(Accepted for publication 12th May, 1952.)

Much evidence has accumulated to show that "neurotropic" viruses can multiply in tissues other than the central nervous system. In fact, since the Poliomyelitis-Coxsackie-Theiler-EMC group of viruses has been shown to be capable of multiplication in tissues of ectodermal mesodermal and endodermal origin, the term "neurotropic virus" now has virtually no meaning.

Less is known, however, about the tissue limitations of the viruses responsible for the arthropod-borne encephalitides. All the members of this group multiply freely in non-nervous tissues of the chick embryo but little has been reported of their ability to multiply in extraneural tissues of post-embryonic animals.

Equine encephalomyelitis, after 26 serial passages in the pad of the foot of guinea-pigs, retains to the full its original infectivity for mice (Olitsky *et al.*, 1934). Hurst (1936) found evidence of slight local multiplication of both Western and Eastern equine encephalomyelitis viruses after inoculation into the pad of foot of guinea-pigs but little or no evidence of local multiplication following intramuscular inoculation of monkeys and guinea-pigs. However, in both these hosts, peripheral inoculation of either of these viruses was followed by the appearance of high level viraemia at a time when no virus was recoverable from the central nervous system: evidence that considerable virus multiplication must have been occurring at some extraneural site.

St. Louis encephalitis virus has been shown to multiply in the spleen of mice (Webster and Clow, 1936) and Lennette and Smith (1939) found that after 12 intratesticular serial passages in mice this virus showed no decrease in virulence when tested intracerebrally. Peck and Sabin (1947), after intravenous inoculation of 10,000,000 intracerebral LD_{50}s of St. Louis encephalitis in 2 to 3-week-old mice, failed to obtain unequivocal evidence of virus multi-

[1] This work was carried out while in receipt of a British Colonial Medical Research Studentship.

plication at any site other than the central nervous system although there is a hint that slight multiplication may occur in the intestinal tract. Schabel (1951) found that when adult hamsters were fed on St. Louis encephalitis and Japanese B encephalitis, both viruses could be recovered from a variety of non-nervous tissues before they appeared in the nervous system, which suggests that there must be an extraneural site of virus multiplication. He was, however, unable to obtain clear evidence of multiplication in any particular tissue.

It was of interest to find whether MVE behaved as a strict neurotrope and if not, to make a comparison between the tissues of young fully susceptible mice and those of older resistant mice with respect to their ability to support virus multiplication.

An investigation of the process of infection in intramuscularly inoculated young (susceptible) mice and older (resistant) mice has shown that in the former there is extensive virus multiplication in muscle or immediately adjacent tissues but that such local multiplication is minimal in older mice.

Materials and Methods.

Virus.

A single pool of MVE virus was used throughout this series of experiments. This pool represented the 11th mouse passage and was prepared from infected 21-day mouse brains harvested 5 days after intracerebral inoculation of a 10^{-1} dilution of MVE Pool 1. (See previous paper in this series). Subsequent preparation was exactly the same as that used for MVE Pool 1 except that ampoules were frozen rapidly in CO_2 ice/alcohol mixture before being stored at —70° C. This pool had a titre of $10^{-5 \cdot 5}$ when inoculated intracerebrally in 28-day mice, and a titre of $10^{-7 \cdot 0}$ when inoculated by the intramuscular route in 10-day mice.

In other respects, materials and methods were exactly the same as those already described in the preceding paper.

EXPERIMENTAL.

Two age groups of mice were used: 5 to 10-day-old mice known to be fully susceptible to peripheral infection with this virus; and 28-day mice, which show a considerable degree of resistance to peripheral infection. Since treatment of these groups was perforce somewhat different, they will be dealt with separately.

(a) *5–10-day mice.*

In each experiment, groups of 5 to 10-day-old mice were inoculated into the gastrocnemius muscle of both hind legs, with a total dose of 0·06 ml. of a 10^{-5} suspension of MVE: this represented a dose of about 200 LD_{50}s per mouse. Four mice were taken from this group at each of the following times after inoculation: ¼ hour, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. At each interval, the 4 mice were anaesthetized and bled out, using sterile precautions, from the great veins of the neck. The whole blood was added to

one drop of sterile 1 p.c. "Liquoid" solution.[2] It had already been found that at this level of concentration, "Liquoid" had no effect on the titre of MVE in blood. The selected tissues were then dissected out in the following order, care being taken to use sterile instruments for removing each piece of tissue. With the mice lying on their dorsal surface, the thorax was first opened and the heart removed. The peritoneal cavity was next opened and the liver, then the spleen and lastly the adrenal were dissected out. Finally, the muscles of the fore limb were removed taking care to exclude the axillary fat and lymph nodes. The mice were then turned over on the ventral surface and the gastrocnemius muscle together with the biceps femoris, semibranosus and semitendinosus muscles were removed taking care to separate them from the sciatic nerve, popliteal lymph nodes and fat deposits in the popliteal and iliac fossae. Popliteal and inguinal lymph nodes and sciatic nerve were next removed. Finally, the brain followed by the spinal cord, were dissected out. In the case of paired tissues, those from both sides of the animals were pooled so that at every time interval, a pool of 4 hearts, 4 spleens, 8 sciatic nerves, etc., was available. Each tissue pool was immediately frozen in CO_2 ice/alcohol mixture and was either thawed at once in 37° C. water-bath or was stored at —70° C. until it could be titrated.

Titrations of the tissue pools were carried out in 11-day eggs, using 2 eggs to each half logarithm dilution. The tissue pools were ground with sterile alundum and taken up in nutrient broth pH 7·6, to which 5,000 units of penicillin and 5,000 units of streptomycin per ml. had been added. A rough estimate of the wet weights of the various tissue pools had previously been obtained by weighing similar pools of normal mouse tissues and on the basis of these weights, sufficient broth was added to give initial suspensions containing 33 p.c. weight by volume of each tissue. These are referred to in the Tables as 10^0 dilutions. In the case of the adrenal glands, sciatic nerve and lymph nodes, the amount of tissue available was so small that it was impossible to prepare such a 10^0 dilution and for these tissues the initial dilutions were about 1 to 10 or 1 to 100 weight by volume suspensions, which were called respectively 10^{-1} and 10^{-2} dilutions. The 10^0 dilution of blood represents whole undiluted blood.

From these initial suspensions, half logarithmic dilutions were prepared in nutrient broth and each dilution was inoculated on the chorioallantoic membrane of 2 eggs using the technique described above. After about 40 hours incubation at 35° C., the chorioallantoic membranes were removed and examined for the presence or absence of pocks. The number of infected and non-infected eggs was recorded and ID_{50} values for each tissue at various times after inoculation were calculated by the method of Reed and Muench (1938).

[2] "Liquoid" is a preparation of sodium polyanethol-sulphonate manufactured by Roche Products Ltd., Welwyn Garden City, England.

The results of a typical experiment are given below:

5-day mice inoculated with 200 LD_{50}s of MVE

Numerator = No. of infected eggs. Denominator = No. of inoculated eggs.
ID_{50} values expressed as the reciprocal of the negative logarithm of the calculated ID_{50}.

1st Sample. Killed 24 hours after inoculation.

Tissue	Reciprocal of negative logarithm of tissue dilution 0·0	0·5	1·0	ID_{50}
Blood	0/2	0/2	0/2	<0·0
Posterior limb muscle	1/2	1/2	0/2	0·2
Anterior limb muscle	0/2	0/2	0/2	<0·0
Brain	0/2	0/2	0/2	<0·0
Spinal cord	0/2	0/2	0/2	<0·0

2nd Sample. Killed 48 hours after inoculation.

Tissue	Reciprocal of negative logarithm of tissue dilution 0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	ID_{50}
Blood	2/2	2/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	3·25
Posterior limb muscle	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	—	2·5
Anterior limb muscle	1/2	0/2	0/2	0/2	—	—	—	—	—	0·0
Brain	0/2	0/2	0/2	—	—	—	—	—	—	<0·0
Spinal cord	0/2	0/2	0/2	—	—	—	—	—	—	<0·0

3rd Sample. Killed 72 hours after inoculation.

Tissue	Reciprocal of negative logarithm of tissue dilution 0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	ID_{50}
Blood	—	—	—	—	2/2	2/2	2/2	0/2	0/2	0/2	3·25
Posterior limb muscle	—	—	—	2/2	2/2	2/2	2/2	1/2	0/2	0/2	3·5
Anterior limb muscle	2/2	2/2	2/2	1/2	0/2	0/2	—	—	—	—	1·5
Brain	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	—	—	2·5
Spinal cord	2/2	2/2	2/2	1/2	0/2	0/2	—	—	—	—	1·5

(b) *28-day mice.*

In the first set of experiments with 28-day mice, the plan used for young mice was followed exactly, i.e. 0·03 ml. of 10^{-5} dilution of MVE was inoculated into each hind leg and tissues from 4 mice were removed, pooled and titrated for virus at various time intervals after inoculation. However, using an inoculum of this strength, which represented less than 10 intracerebral LD_{50}s for mice of this age, no virus could be detected in any of the tissues tested up to 8 days after inoculation.

In view of this finding, a rather different plan was adopted for investigating virus multiplication in these older mice. The results of the age resistance experiments had suggested the possibility that at 28 days of age, there might be considerable individual variation in susceptibility to peripheral inoculation with MVE. It was therefore considered advisable to select only two important

tissues and to make quantitative estimates of their virus content in individual mice at different times following virus inoculation. Accordingly, 12 mice were inoculated into the gastrocnemius muscle of each hind leg with 0·03 ml. of a 10^{-2} dilution of MVE; this represents a dose of approximately 2,000 intracerebral LD_{50}s for mice of this age and is 1,000 times more concentrated than the dose given to 5 to 10-day mice. Using post-inoculation time intervals similar to those used in the experiments on young mice, 2 mice were killed at each time interval, and blood and hind limb muscle were taken separately from each one. Each sample of blood and muscle was titrated separately in eggs, using the technique already described in the section on 5 to 10-day mice.

RESULTS.

(a) *5–10-day mice.*

The outstanding features shown in Tables 1 and 2 and in Fig. 1, are the active multiplication of virus at the inoculation site; high-level viraemia from the second to the fourth day; and multiplication of virus in the central nervous system from the third day onwards.

The findings in other tissues are hardly sufficient to warrant discussion. There is a hint that multiplication may have occurred in lymph nodes, adrenal, and distant muscle but without any knowledge of the blood content of the tissue emulsions and of possible mechanisms by which these tissues may have accumulated virus from the blood, no certainty can be possible.

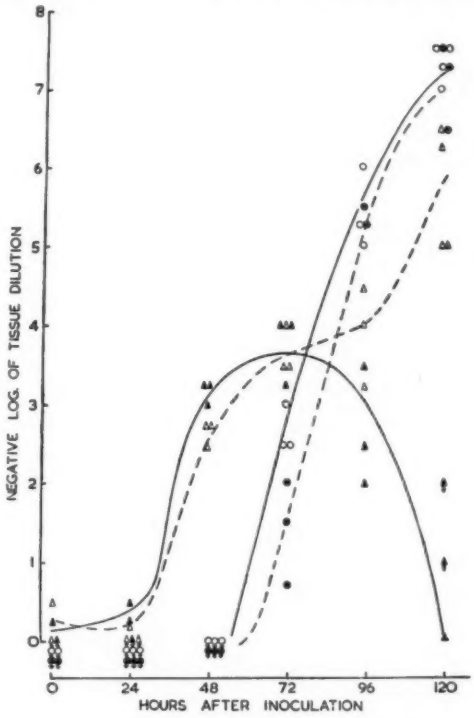

Fig. 1. Murray Valley encephalitis in mice. Multiplication of virus inoculated into the hind legs of 5–10-day-old mice.

Dose: 0·06 ml. of a 10^{-5} suspension of MVE per mouse.

▲——▲ Blood.
△——△ Posterior limb muscle.
O——O Brain.
●——● Spinal cord.

As in the majority of experiments of this type, very little virus was recoverable during the first 24 hours after inoculation. At 48 hours, active multipli-

cation was obviously occurring in the hind limb muscles, and a considerable viraemia had developed. It is noteworthy, however, that at this period no virus was detected in any of the nervous tissues.

TABLE 1.

Murray Valley encephalitis virus in tissues of 5–10-day-old mice.

Dose = *ca.* 200 intracerebral LD_{50}s per mouse.

Tissue	Time after Inoculation. ¼ hour	24 hours	48 hours	72 hours	96 hours	120 hours
Blood	<0;0·25	0·5;0·25;<0	3·0;3·25;3·25	4·0;4·0;3·25	2·0;3·5;2·5	<2·0;<1·0;0·0
Posterior limb-muscle	<0;0·5	<0;<0;0·2	2·75;2·75;2·5	4·0;3·5;3·5	4·0;4·5;3·25	6·5;6·25;5·0;5·0
Anterior limb-muscle	—	<0	0·0	1·5	1·75	2·25;2·5
Brain	<0;<0	<0;<0;<0	<0;<0;<0	2·5;3·0;2·5	5·0;6·0;5·25	7·0;7·5;7·5;7·25
Spinal cord	<0;<0	<0;<0;<0	<0;<0;<0	2·0;0·75;1·5	5·5;5·25	7·5;6·5;7·25
Sciatic nerve	<2·0	<2·0	<2·0	2·25	3·0	5·0

Figures represent the reciprocal logarithm of the highest tissue dilution in which virus was detectable.

These values were obtained from a series of experiments involving the use of 5 separate batches of mice.

Each figure is obtained by using tissue pools from 4 mice.

TABLE 2.

Murray Valley encephalitis virus in tissues of 5–10-day mice.

Dose: *ca.* 200 intracerebral LD_{50}s per mouse.

Tissue	Time after inoculation. ¼ hour	24 hours	48 hours	72 hours	96 hours	120 hours
Adrenal	<1·5	<1·5	<1·5	2·5	2·5	<2·0
Spleen	<0·0	<0·0	2·0	2·5	1·5	<1·0
Heart	<0·0	<0·0	2·0	<2·0	1·0	1·5
Lymph node	<1·0	<1·0	2·75	1·75	1·5	<1·0
Liver	<0·0	<0·0	<1·5	<2·0	<1·0	1·0

All mice inoculated on 2/7/51.

The figures represent the reciprocal logarithm of the highest tissue dilution in which virus was detectable.

Each figure is obtained by using tissue pools from 4 mice.

Seventy-two hours after inoculation, virus had appeared in the brain, spinal cord and sciatic nerve and had reached its maximal concentration in the blood (10^{-4}).[3] The virus concentration in inoculated muscle had risen to a similar value and the concentration in muscle remote from the inoculation site had risen 10-fold to a level of $10^{-1.5}$.

[3]This and subsequent figures mean that 0·05 ml. of a 10^{-4} tissue dilution infected 50 p.c. of inoculated eggs.

At 96 and 120 hours, high concentrations of virus were present in brain, spinal cord and sciatic nerve. Virus concentration in the hind limb muscles had also increased and at 120 hours values of 10^{-5} and 10^{-6} were obtained. From 72 to 120 hours uninoculated muscle contained significant amounts of virus but this can hardly be interpreted as evidence of virus multiplication there. The concentration of virus in the blood had fallen nearly to zero on the 5th day.

TABLE 3.

Murray Valley encephalitis virus in tissues of 28-day mice.

Dose: *ca.* 2,000 intracerebral LD_{50}s per mouse.

Tissue	Time after inoculation. ¼ hour	24 hours	48 hours	72 hours	96 hours	120 hours
Blood.						
Mouse 1	<0·0	<0·0	<0·0	<0·0	1·0	0·0
2	<0·0	<0·0	0·5	0·0	<0·0	<0·0
Muscle.						
Mouse 1	>1·0	0·25	0·0	1·25	2·5	0·5
2	>1·0	0·0	1·25	1·73	0·5	<0·0
Brain.						
Mouse 1	<0·0	<0·0	<0·0	<0·0	<0·0	<0·0
2	<0·0	<0·0	<0·0	<0·0	<0·0	<0·0
Spinal cord.						
Mouse 1	<0·0	<0·0	<0·0	<0·0	<0·0	<0·0
2	<0·0	<0·0	<0·0	<0·0	<0·0	<0·0

Figures represent the reciprocal logarithm of the highest tissue dilution in which virus was detectable.

Each figure is obtained using tissue from a single mouse.

Up to 120 hours after inoculation with 200 LD_{50}s of MVE virus, no clinical symptoms of illness were apparent in these 5 to 10-day-old mice. On the basis of results obtained in other experiments it would be expected that mice of this age inoculated with this amount of virus would fall ill and die on the 6th and 7th day.

(b) *28-day mice.*

The picture is significantly different in older mice. Taking first the site of intramuscular inoculation, virus was present 15 minutes after inoculation, and was detectable, at its lowest level, at 24 hours. There was a distinct increase during the 2, 3-to-4-day period but the concentration of virus never approached the level reached at this site in suckling mice. A low grade viraemia was detectable in 4 out of 8 mice over the period from 2 to 5 days. At no stage was virus detectable in the central nervous system (see Table 3).

DISCUSSION.

It seems, then, that with increasing age mice show no sharp or qualitative change in the process of peripheral infection. As mice become older, tissue at

the inoculation site (probably, though not certainly, muscle tissue) becomes progressively less capable of supporting virus multiplication, though even at the age of 28 days some multiplication occurs.

The degree of viraemia is probably dependent on the local production of virus, although in the earlier stages of the disease the blood of suckling mice shows a higher level of virus than does the inoculated muscle. No evidence has been obtained to explain the fact that on the 5th day of the disease, when virus is still being produced in large quantities in the inoculated tissues, no virus is recoverable from the blood but this may be a result of the presence of neutralizing antibody.

In the first paper of this series it was pointed out that with increasing age, there was a progressive decline in the proportion of mice developing encephalitis following peripheral inoculation of virus. Results given in this paper suggest that this phenomenon may be related both to the lower average concentration of virus in the blood of the older animals and to the increasing resistance of some local "barrier" similar to those postulated by Sabin and Olitsky (1937 a, b; and 1938 a, b).

SUMMARY.

The virus concentration of various tissues following intramuscular inoculation of MVE, has been studied in suckling and in 4-week-old mice.

In suckling mice, early and extensive multiplication occurs in the inoculated muscle and a marked viraemia develops which precedes active multiplication in the central nervous system.

There is no clear evidence for or against multiplication in several other tissues.

In 4-week-old mice, local multiplication and viraemia are at a much lower level, and invasion of the central nervous system does not occur.

The processes concerned are briefly discussed.

Acknowledgment. In the preparation of this paper, the author is much indebted to the help and criticism of Sir Macfarlane Burnet. Miss Mary Cook kindly prepared Fig. 1.

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LYSOGENICITY AND LYSIS PATTERNS IN THE SALMONELLAS

I. *S. ADELAIDE*, *S. WAYCROSS* AND *S. BOVIS-MORBIFICANS*

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The occurrence of lysogenic strains of Salmonellas has been recognized for many years and was early emphasized by Burnet (1932). Recently Boyd (1950) has investigated the symbiotic phages of *S. typhi-murium* and classified a number of them, using Burnet's original grouping into A and B types.

The present work consists of an investigation for lysogenicity of a number of Salmonella types, especially those which are more peculiar to Australia and of which we, being the Salmonella typing centre, have a large collection of local strains. The types presented here are *S. adelaide*, which was first isolated here and of which we have a unique collection, *S. waycross*, of which we have the only large collection from an outbreak at Atherton in Queensland, and *S. bovis-morbificans*, which, next to *S. typhi-murium*, is the commonest cause of human salmonellosis in Australia.

A number of lysogenic strains were found and their phages isolated and examined. The characteristics of these phages will be recorded in a separate series of papers. The object of the present series is to emphasize the differences in lysogenicity and lysis patterns of the various Salmonella types.

METHODS.

Detection of lysogenicity. Several methods were used and the tests were made on the homologous type only using groups of strains.

The cross-plating method of Fisk (1942) using every combination of pairs of strains in a group was most satisfactory and was regularly used. Generally 4 hours broth cultures provided the inocula, but older cultures were sometimes used. Frequently phage action was not obvious on the superimposed spots but phage, if present, was easily detected when the underlying agar and mixed growth was emulsified in broth, centrifuged and the supernatant tested on the two strains of the mixture and an indicator strain if available. When only one

[1] This work was partly supported by a grant from the National Health and Medical Research Council, Canberra.

of the pair of strains in the mixture was lysogenic it was usually recognized by its resistance to lysis by the supernatant.

Mixed growth in broth using pairs of strains and incubating for 4 hours or 20 hours at 37° C., was used to examine some groups. The mixed culture was centrifuged and the supernatant tested on each strain originally present and also on a suitable indicator strain if available.

All agar cultures were examined for signs of lysis after the usual period of incubation (2 hours at 37° C., overnight at 4° C., followed by a further 4 hours incubation at 37° C.), and further tested by digging out some of the agar and mixed growth, emulsifying in broth, centrifuging and testing the supernatant on each of the original strains.

Occasionally broth filtrates of 4 hours and 18 hours cultures were tested for phage action on suitable indicator strains.

The detection of possible phage-carrying strains was facilitated after some phage preparations had already been obtained from a given group. By testing the group with each phage preparation, resistant strains were detected. These were suspected of being lysogenic and harbouring the phage to which they were resistant. An intensive search for the phage was then made in these organisms by various methods, using, where possible, known susceptible, nonlysogenic strains as indicators.

Investigation of lytic action. A lysogenic strain was grown with a suitable indicator strain on nutrient agar, the lysed patch was extracted in broth, centrifuged, and the supernatant propagated in broth with the indicator strain for a number of transfers. The final broth culture was filtered or centrifuged and heated at 56° C. for 30 minutes to free it from living bacteria. The nature of the lytic action was then investigated on the indicator strain on nutrient agar using a series of 10-fold dilutions. Any plaques formed were carefully examined and described.

Isolation of phages. A suitable dilution of the broth preparation mixed with a 4-hours broth culture of a suitable indicator strain was plated out on agar, and incubated in the usual way. Separate plaques were picked off, emulsified in broth, and again plated out. Separate plaques were again picked off and this process repeated several times.

The nutrient broth used throughout this work was made from fresh ox-heart extract by the method of Wright. The nutrient agar was made from this broth by addition of 1·2 p.c. agar. Other types of broth or agar did not give typical lysis which was often difficult even to detect.

RESULTS.

S. adelaide.

Forty-eight of the fifty strains previously described by Atkinson *et al.* (1952) were tested. Strains 29 and 44 were lost before the phage work began. Lytic combinations were readily detected by the methods of cross-plating or mixed growth in broth.

Twelve lysogenic strains were found. Of these, 10 strains (13, 15, 16, 37, 39, 40, 43, 45, 47, 49) gave similar lysis consisting of two types of plaques, hazy and clear. The clear plaques had regular, well-defined edges and no secondary growth. The hazy plaques, which far outnumbered the clear ones, had irregular edges and diffuse secondary growth. The other two lysogenic strains (2 and 20) each produced only one type of plaque which was clear with a somewhat irregular edge and a small spot of secondary growth in the centre.

For further investigation of the phages, strains 2, 20 and 37 were cross-plated on strain 14 which was non-lysogenic and susceptible to the phages so far found. The phages were isolated and propagated on this strain for several sub-cultures after which they produced numerous plaques or confluent lysis at dilutions of 10^{-3} to 10^{-4}. With these preparations, which were labelled Ad2, Ad20, Ad37 C (clear plaque) and Ad37 H (hazy plaque), the 48 strains of *S. adelaide* were tested. The results of these tests are given in Table 1.

TABLE 1.

Action of four S. adelaide *phage preparations on 48 strains of* S. adelaide.

Strain No.	Phage preparation Ad2	Ad20	Ad37C	Ad37H	Strain No.	Phage preparation Ad2	Ad20	Ad37C	Ad37H
1	+	+	+	+	28	+	+	+	+
* 2	—	±	+	+	30	+	+	+	+
3	+	+	+	+	31	+	+	+	+
4	+	+	+	+	32	±	—	—	—
5	+	+	+	+	33	+	+	+	+
6	+	+	+	+	34	+	+	+	+
6	+	+	+	+	35	+	+	+	+
7	+	+	+	+	36	+	+	+	+
8	+	+	+	+	*37	+	+	—	—
9	+	+	+	+	38	+	+	+	+
10	+	+	+	+	*39	+	+	—	—
12	+	+	+	+	*40	+	+	—	—
*13	+	+	—	—	41	+	+	+	+
14	+	+	+	+	42	+	+	+	+
*15	+	+	—	—	*43	+	+	—	—
*16	+	+	—	—	*45	+	+	—	—
17	+	+	+	+	46	+	+	+	+
18	+	+	+	+	*47	+	+	—	—
19	+	+	+	+	48	+	+	+	+
*20	+	—	+	+	*49	+	+	—	—
21	+	+	+	+	50	+	+	+	+
23	+	+	+	+	51	+	+	+	+
25	+	+	+	+	52	+	+	+	+
26	+	+	+	+	53	+	+	+	+
27	+	+	+	+					

The phage preparations were used at a concentration 10 × the C.T.D.
* = Lysogenic strain.
+ = Confluent lysis.
± = Semi-confluent lysis or very numerous plaques.
— = No lysis.

All the non-lysogenic strains except strain 32 were lysed by all the phages. The lysogenic strains were resistant to the phage they carried but susceptible to the other phages. Strain 32 was suspected of carrying phage on account of its resistance to the action of Ad2, Ad37 C and Ad37 H. Extensive tests, however, failed to reveal any lysogenic action.

S. waycross.

There were 31 strains of this type of which 30 were described by Atkinson *et al.* (1950) and consisted of 29 strains from a single outbreak of gastro-enteritis in Queensland and 1 strain (Cronin) from a South Australian case. The other single strain (S 215) was the original culture described as a new type, *S. waycross*, by Seligmann and Saphra (1948) and came from a case of chronic cystitis. We are grateful to Dr. Seligmann for sending us this culture.

Only two lysogenic strains, Cronin and S 215, were detected. Both showed the same kind of lysis consisting of two rather similar plaques, one showing a large spot of secondary growth in a clear plaque and the other clear with slight or no secondary growth. The plaques with secondary growth predominated. The Cronin phages were propagated and isolated on Currie, one of the Queensland strains. The purified preparations, which were labelled W Cronin A (clear plaque) and W Cronin B (secondary growth plaque) and produced confluent lysis at a dilution of 10^{-3} to 10^{-4}, were tested on the 31 strains. The 29 Queensland strains were lysed by both phages. Cronin and S 215 were not lysed by either phage preparation. The S 215 phages were also isolated and propagated on Currie and gave the same reactions as the Cronin phages when tested on the 31 *S. waycross* strains.

S. bovis-morbificans.

Over 300 local strains of this type were available. Sixty representative strains were selected and tested in groups of twenty. Cross-plating and mixed broth tests on the first group of twenty strains (1–20) showed that every strain was lysogenic. In the second group of twenty strains (21–40) all except strain 36 were lysogenic. The third group of twenty strains (41–60) were investigated by cross-plating on strains 17, 18 and 36. These were selected because strains 17 and 18 were susceptible to most of the phages already tested, and strain 36 was the only non-lysogenic strain so far found. By these tests all in this group were lysogenic, and indications of lytic action due to strain 36 were also obtained.

As all the 60 strains so far tested were lysogenic, the type of lysis due to a given strain was investigated by growing that lysogenic strain either with strain 18, which was highly susceptible to all the phages so far found, or with some other susceptible strain to whose phage the lysogenic strain was resistant. In these combinations the lysis was due to the phages of the lysogenic strain under investigation, as tests of such mixtures showed no lytic action on this strain, but obvious lytic action on the other strain (subsequently referred to as "propagating strain").

Ten phage preparations were made and labelled with the number of the lysogenic strain followed by the number of the propagating strain thus:

BM 7/18, BM 13/5, BM 14/18, BM 17/18, BM 19/18, BM 35/18, BM 45/18, BM 46/18, BM 48/18, BM 53/18.

These preparations produced at least four types of lysis. BM 7/18, BM 19/18, BM 35/18 and BM 48/18 all showed at least two kinds of plaques, one medium size with a spot of secondary growth, the other clear and small; the former predominated. BM 17/18, BM 46/18 and BM 53/18 produced small clear plaques and BM 13/5 and BM 45/18 produced two types of small plaques, one clear and the other hazy. These plaques resembled those of the *S. adelaide* phages Ad37 C and H. BM 14/18 produced only medium plaques with a spot of secondary growth similar to those produced by the BM 19/18 group but unaccompanied by other plaques.

The results of testing these phage preparations on the group of strains of *S. bovis-morbificans* from which they were derived, are given in Table 2.

TABLE 2.

Action of 10 S. bovis-morbificans *phage preparations on the strains of* S. bovis-morbificans *from which they were obtained.*

Strain No.	Phage preparations 7/18	13/5	14/18	17/18	19/18	35/18	45/18	46/18	48/18	53/18	Strain group.
7	—	—	—	—	—	—	—	—	—	—	Group 1
13	+	—	+	—	+	+	—	—	+	—	Group 3
14	+	+	—	±	+	+	+	±	+	±	Ungrouped
17	+	+	+	—	+	+	+	—	+	—	Group 2
19	—	—	—	—	—	—	—	—	—	—	Group 1
35	—	—	—	—	—	—	—	—	—	—	Group 1
45	+	—	+	—	+	+	—	—	+	—	Group 3
46	+	+	+	—	+	+	+	—	+	—	Group 2
48	—	—	—	—	—	—	—	—	—	—	Group 1
53	+	+	+	—	+	+	+	—	+	—	Group 2

+ = confluent or semi-confluent lysis. ± = plaques. — = no lysis.

Three groups of strains emerged from these tests and the grouping is indicated in the Table. Group 1 consisted of strains 7, 19, 35 and 48 which were not lysed by any phage preparation and readily yielded phages themselves. The phages obtained from this group all gave the same type of lysis (see above). Group 2 consisted of strains 17, 46 and 53 which were lysed by all the phage preparations except those derived from these strains. These were the phages producing the small clear plaques. Group 3 consisted of strains 13 and 45 which were not lysed by their own phages nor by the phages of Group 2 but were lysed by all the other phages in Table 1. The phages from this group produced clear and hazy plaques. Strain 14 resembled no other strain and was lysed by all phages except that derived from itself. No group has yet been allotted to this strain as its relationships are obscure. The phage obtained from it produced only the secondary growth type of plaque.

To investigate the occurrence of these groups twenty more strains of *S. bovis-morbificans* were selected from the original sixty and tested with the ten phage preparations. The results are given in Table 3.

TABLE 3.

Action of 10 S. bovis-morbificans *phage preparations on 13 strains of* S. bovis-morbificans.

Strain No.	Phage preparations. 7/18	13/5	14/18	17/18	19/18	35/18	45/18	46/18	48/18	53/18	Strain group.
3	+	+	+	—	+	+	+	—	+	—	Group 2
5	+	+	+	—	+	+	+	—	+	—	Group 2
10	—	—	—	—	—	—	—	—	—	—	Group 1
15	+	+	+	—	+	+	+	—	+	—	Group 2
18	+	+	+	±	+	+	+	±	+	±	Group 4
26	+	—	+	—	+	+	—	—	+	—	Group 3
32	+	+	+	—	+	+	+	—	+	—	Group 2
33	+	—	+	—	+	+	—	—	+	—	Group 3
36	+	+	+	±	+	+	+	±	+	±	Group 4
49	—	—	—	—	—	—	—	—	—	—	Group 1
50	+	—	+	—	+	+	—	—	+	—	Group 3
54	+	+	+	±	+	+	+	±	+	±	Group 4
57	—	—	—	—	—	—	—	—	—	—	Group 1

+ = confluent lysis. ± = plaques. — = no lysis.

Seven of the twenty strains tested (34, 41, 44, 51, 52, 56 and 58) did not give satisfactory reactions and were not included in Table 3. Of the remaining 13 strains 10 fell in Group 1, 2, or 3 and the other three strains (18, 36 and 54) were placed in a new Group 4. These strains were lysed by all the phages but failed to yield a satisfactory phage preparation themselves owing to their ready lysis by other lysogenic strains. To obtain phage representative of this group a susceptible non-lysogenic strain is required but has not yet been found. Nevertheless, Group 4 is easily recognized by its general susceptibility to lysis.

The phage preparations 17/18, 46/18 and 53/18 were weaker than the others and most restricted in action. They produced isolated plaques only on Group 4 strains and strain 14 of Table 2.

As none of the phage preparations were purified the groupings suggested here are only tentative. However, from these results the complexity of the lysis and lysogenicity pattern of *S. bovis-morbificans* is obvious and the results of further investigation of this group will appear in a separate paper.

DISCUSSION.

The differences in lysogenicity pattern of the three Salmonella types were marked. Almost every strain of *S. bovis-morbificans*, like *S. typhi-murium*, was lysogenic but only about 25 p.c. of *S. adelaide* and few *S. waycross* strains were lysogenic.

Generally in any group the lysogenic strains were resistant to the phage they carried. Thus resistance to lysis often indicated lysogenicity and lysis by a phage usually indicated absence of that phage from the susceptible strain which might still however carry other phages. In some strains resistance to lysis was not associated with carriage of phage. Thus in the *S. adelaide* series strain 32 was weakly lysed by Ad 2 phage but was resistant to the other phages. Extensive tests for lysogenicity were carried out but no phage was detected. This strain was the only one among the 48 tested whose lysogenicity and lytic patterns were not related. All the other strains were either non-lysogenic and lysed by all the phages, or lysogenic and resistant to the phage which they carried, but susceptible to the other phages. The *S. waycross* group, on the other hand, showed no resistant non-lysogenic strains. The two lysogenic strains were resistant to both phage preparations and the rest of the strains, all of which were not lysogenic, were lysed by both phages. With *S. bovis-morbificans* the lysis pattern was complicated by the frequent occurrence of lysogenicity, one type of which rendered the organisms (Group 1) resistant to all the phages so far found. On the other hand certain strains (Group 4) though lysogenic, were lysed by all the phages tested here. From these strains no suitable phage preparation has yet been obtained because of their ready lysis by all other strains. Further work is in progress with purified phages and should clarify the lysis pattern in this group.

Considerable variation in the appearance of the lysis occurred and a number of strains produced more than one type of plaque. They probably carried more than one type of phage. Thus ten of the twelve lysogenic strains of *S. adelaide* produced two types of plaques possibly from two different phages. Both the lysogenic strains of *S. waycross* produced two somewhat similar plaques which probably arose from the one type of phage. The lysis produced by Group 1 strains of *S. bovis-morbificans* showed two distinct plaques, and Group 3 strains also showed two kinds of plaques resembling those of the common lysogenic type of *S. adelaide*. Judging by plaque appearances one lysogenic type of *S. waycross*, at least two different lysogenic types of *S. adelaide* and at least four different lysogenic types of *S. bovis-morbificans* were found.

The patterns of lysis and lysogenicity may be used to group the strains within a type and so-called phage-typing has usually depended upon the lysis pattern with a series of phage preparations such as the Vi phages for *S. typhi* typing. However, the lysogenicity pattern of the strain itself has also been used, as with *S. typhi-murium* (Boyd, Parker and Mair, 1951) and *S. paratyphi B* (Scholtens, 1950). The Salmonellas we have investigated here may be grouped by phage methods. The grouping of *S. adelaide* could best be based on patterns of both lysis and lysogenicity and the method will be further developed in a separate paper. Lysis or lysogenicity could be used to type *S. waycross*, of which all strains from the Atherton outbreak of enteritis were similar types

being non-lysogenic and susceptible to the two *S. waycross* phages, but the two lysogenic strains were of entirely different origin, each coming, however, from a chronic type of illness. Detailed description of the *S. waycross* phages will be reported separately. The complex lysis and lysogenicity patterns of *S. bovis morbificans* lend themsleves well to the development of a method of grouping strains of this organism. However, a discussion of this method goes far beyond the scope of this paper and will be given in a separate paper embodying the results of further work on the phages of *S. bovis-morbificans.*

SUMMARY.

Forty-eight strains of *S. adelaide*, thirty-one strains of *S. waycross* and sixty strains of *S. bovis-morbificans* were examined for lysogenicity. Twelve strains of *S. adelaide*, two strains of *S. waycross* and sixty strains of *S. bovis-morbificans* proved lysogenic.

Among the lysogenic strains two distinct types of lysis occurred for *S. adelaide*, one type for *S. waycross* and at least four types for *S. bovis-morbificans.*

The lysis pattern of the strains within a type was usually related to lysogenicity so that phage carriers were generally resistant to lysis by phage which they carried but were lysed by other phages.

Tentative groupings by lysis and lysogenicity are suggested for these three Salmonella types.

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AN ENCEPHALITIS VIRUS ISOLATED IN SOUTH AUSTRALIA

I. SOME CHARACTERISTICS OF THE VIRUS

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Early in 1951 an outbreak of encephalitis occurred in the Murray Valley from which a virus was isolated. The course of the epidemic, the clinical picture and the virus have been described by the Melbourne workers, Anderson, S. G. (1952), French, E. L. (1952), Robertson, E. G. and McLorinan, H. (1952), Robertson, E. G. (1952), Anderson, S. G., Donnelley, M., Stevenson, W. J., Caldwell, N. J. and Eagle, M. (1952). At the same time a few sporadic cases of encephalitis occurred in South Australia. From the brain of a fatal case a virus was isolated (Miles, Fowler and Howes, 1951). In this paper a description is given of some of the characteristics of this virus.

Materials and Methods.

All work has been done on virus isolated from one case. When material from an early passage was desirable, freeze-dried 4th mouse passage material either immediately or after one further mouse passage was used. For certain purposes a more thoroughly mouse adapted strain between the 15th–30th mouse passage has been employed. The virus was normally passed intracerebrally in weaned mice and portions of a 20 p.c. brain extract in 10 p.c. rabbit serum saline were stored in hard glass ampoules in dry ice. No significant fall in titre of this material occurred in two months' storage, but after six months there had been a one to two log fall in titre.

Inoculations.

Intracerebral inoculation under ether anaesthesia was the method most commonly employed. Penicillin and streptomycin were added to the inoculum to make a final concentration of each antibiotic of 500 units per ml. The dose inoculated varied with the type of animal to be inoculated.

Intranasal inoculations were carried out under ether anaesthesia in a protective hood.

Other peripheral inoculations were normally given intramuscularly into the leg.

EXPERIMENTAL.

Disease in weaned mice.

The disease as seen in weaned mice after intracerebral inoculation of moderate doses of virus usually gave a very characteristic clinical picture. Most

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mice began to appear sick on the fourth day after inoculation when they showed a ruffled coat, sat rather hunched up and moved slowly. From time to time they would have violent convulsions which left them considerably weakened, but were never observed to be fatal. Many showed definite hind limb paralysis late on the fourth day and were dead by the fifth, but in some the disease progressed more slowly and they might survive until the sixth day. Paralysis was of the ascending type most commonly seen when the arthropod-borne encephalitides are inoculated intracerebrally into mice.

With large doses of virus inoculated intracerebrally death might occur as early as the fourth day and with minimal doses on the eighth or ninth. Few deaths attributable to the virus occurred later than the ninth day. The LD_{50} for the virus inoculated by this route was normally contained in 0·03 ml. of a dilution between 10^{-7} and 10^{-8}.

The disease was not greatly different when animals were inoculated by other routes, but the incubation period differed and the infective titre for weaned mice was much lower (Table 1). When the virus was inoculated intranasally the titre was 3·8 logs lower than the same material inoculated intracerebrally and when inoculated intramuscularly 3·1 logs lower.

TABLE 1.

Parallel titrations in mice of virus inoculated by different routes.

Route of inoculation	Weaned or suckling mice	Dilution of virus and mouse deaths								Log titre*
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Intramuscular	Suckling	—	—	—	—	—	5/6	1/6	0/6	7·7
Intramuscular	Weaned	3/5	4/5	4/5	1/5	1/5	0/5	0/5	—	4·6
Intranasal	Weaned	—	3/5	2/5	0/5	0/5	0/5	—	—	3·9
Intracerebral	Weaned	—	—	—	5/5	5/5	3/5	2/5	—	7·7

* The titre is expressed as the log of the reciprocal of the dilution in which a volume of 0·03 ml. contained one LD_{50} of virus.

Numerator = Mouse deaths. Denominator = Number of mice in group.

Intranasal inoculation, like the intracerebral, gave good clear-cut endpoints although there was a longer spread over the period during which the mice died. Deaths have normally occurred between the sixth and the twelfth day. Titration by the intramuscular route was very untidy and the endpoint was extremely unsatisfactory, since not all mice died even when large doses of virus were used and there were occasional deaths using dilutions as high as 10^{-6}. Altogether in the experiments using virus at a dilution of 10^{-2} and twenty-one-day-old mice 21 out of 35 mice died. The day of death of mice dying with a clinical picture suggestive of infection with this virus varied between the sixth to the nineteenth day.

Susceptibility of suckling mice.

In these experiments suckling mice were usually avoided because Coxsackie viruses were being used in the same laboratory and the possibility of contamination made it seem desirable to avoid their use when the use of weaned mice was practicable, but a few experiments were made to give some indication of the susceptibility of suckling mice to this virus.

TABLE 2.

Deaths of mice at different ages after peripheral inoculation.

Age of mice in days	Dilution of virus	Number in group	Day of death	Number of deaths	Number of survivors	Average day of death of those dying
1	1:5	6	6 × 6*	6	0	6
3	1:5	4	5 × 2, 6, 8	4	0	6
7	1:5	5	5 × 2, 6 × 3	5	0	5·6
14	1:5	6	6 × 2, 7 × 2, 8, 9	6	0	7·2
21	1:5	4	7, 8 × 2, 11	4	0	7·9
	1:50	30	6, 8 × 2, 9 × 3 10 × 6, 11 × 3, 12 × 2, 17	18	12	10·2
28	1:5	4	6, 7 × 2, 9	4	0	7·3
	1:50	30	10 × 3, 12 × 2, 13, 16, 17 18, 19	10	20	13·7

* The figure given after the multiplying sign represents the number of animals dying on any one day when that number was greater than one.

Normally suckling mice inoculated intracerebrally with virus died one day earlier than weaned mice inoculated with the same material. The titre of material after intracerebral inoculation appeared to be slightly higher in suckling than weaned mice, but not more than tenfold. The intramuscular and intracerebral titres in 48-hour-old mice were not greatly different, but the intracerebral titre was usually rather the higher. In the experiment shown in Table 1 the intramuscular titre in suckling mice was the same as the intracerebral in weaned mice. Other experiments suggest that usually the titre in suckling mice is slightly higher. Usually in 48-hour-old mice inoculated intramuscularly death occurred on the fifth or sixth day with large doses of virus and between the eighth and tenth day with minimal doses.

Suckling mice were fully susceptible to this virus either intracerebrally or intramuscularly. Weaned mice of the inbred strain bred in this Institute were fully susceptible intracerebrally, but showed a substantially lower susceptibility to peripheral inoculation.

The development of resistance to peripheral inoculation.

The data given above show that the strain of mice used showed substantially greater resistance to peripheral inoculation at the age of 21 days than at 48 hours. A few experiments were done to indicate the age at which this resistance developed. We obtained no evidence of a substantial alteration in resistance between the ages of one and seven days, but by the fourteenth day the incubation period after peripheral inoculation of large doses of virus was increasing (Table 2). The use of such large doses of virus did not indicate any further substantial variations up to the twenty-eighth day, but when smaller doses of virus were used evidence was obtained of a steadily increasing resistance.

Virus in the blood stream.

After intracerebral inoculation of large doses of virus into weaned mice, sufficient virus was present in blood to kill four out of four weaned mice inoculated intracerebrally with heparinized blood taken 24 and 48 hours after inoculation. One of four mice receiving 72-hour blood died with typical signs of infection with the virus. Blood taken on the fourth day when the inoculated mice were beginning to show signs of disease, did not contain sufficient virus to infect weaned mice when given intracerebrally (Table 3).

TABLE 3.

Virus in blood of different animals. Species of animal tested and number of mouse deaths after I.C. inoculation of blood.

Day	Mouse	Lambs	Horse	Chicks	Cockerels		Pigeons		Gulls		Ducks	
					1	2	I.C.	I.M.	1	2	1	2
1	4/4	N.T.	N.T.	4/4	4/4	0/4	N.T.		4/4	4/4	4/4	4/4
2	4/4	N.T.	N.T.	4/4	2/4	0/4	N.T.		4/4	4/4	4/4	4/4
3	1/4	N.T.	N.T.	3/4	0/4	0/4	4/4	0/4	4/4	4/4	3/4	4/4
4	0/4	N.T.	N.T.	1/4	0/4	0/4	N.T.		4/4	4/4	1/4	Dead
5	All mice dead	0/4	N.T.	0/4	0/4	0/4	N.T.		4/4	4/4	0/4	—
6		N.T.	N.T.	0/4	0/4	0/4	0/4	0/4	4/4 Died	4/4	0/4	
7		Died	N.T.	0/4	0/4	0/4	N.T.			N.T.	0/4	
8			N.T.	0/4	0/4	0/4	N.T.			1/4	0/4	
9			0/4	0/4	0/4	0/4	0/4	0/4		Dead	0/4	

Numerator = Mouse deaths. Denominator = Number of mice in group.
N.T.—Not tested. I.C. = Intracerebral inoculation. IM = Intramuscular inoculation.
No virus was found in any group later than the ninth day after inoculation.

Rats.

Hooded rats of the strain bred in this Institute when between 21 and 28 days old showed no evidence of illness after inoculation with virus. However, after 28 days specific complement fixing and neutralizing antibodies were found in the serum.

The susceptibility of rats at different ages to intracerebral inoculation was tested. Suckling rats inoculated at three and at four days old died from the sixth to the ninth day. Their brains contained large amounts of virus at the time of death and uninoculated litter mate controls remained healthy. There were no deaths among rats inoculated when seven, sixteen or twenty-one days old. One of the seven-day animals appeared to be ill for several days and although about the same size as the others at the beginning of the experiment did show marked stunting at the end.

Rabbits.

Young rabbits were inoculated intracerebrally with 0·3 ml. of virus under ether anaesthesia. They showed no ill health, nor any pyrexia (temperatures were taken twice a day for three weeks). However, they developed high titre neutralizing and complement fixing antibodies against the virus.

Guinea-pigs.

Recently weaned guinea-pigs when inoculated intracerebrally with 0·1 ml. of concentrated virus suspension showed no signs of ill health, but in all cases on the fourth day the temperature rose over 104° F. and sometimes remained raised on the fifth day also (see Fig. 1). Complement fixing antibodies appeared in the blood in the second or third week and neutralizing in the third or fourth week and reached a high titre.

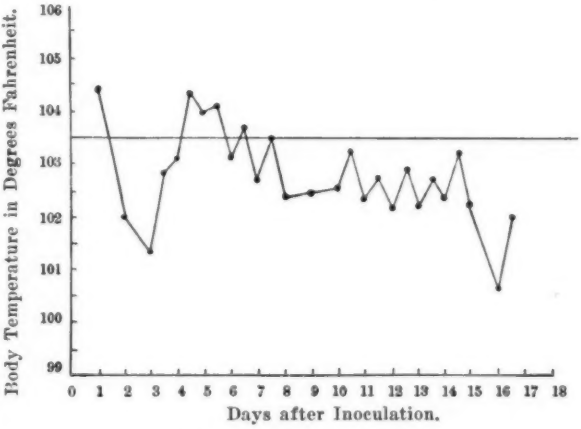

Fig. 1. Temperature chart of guinea-pig inoculated intracerebrally with 20 p.c. virus suspension.

Sheep.

Four 48-hour-old lambs were inoculated intracerebrally with 0·5 ml. of fourth-passage infected mouse brain in a 20 p.c. suspension. On the fourth day after inoculation all showed raised temperatures varying between 105·9 and 107·4° F. On the next day the temperatures varied between 104·7 and 106·6° F. and three of the four were showing signs of ill health. On the next day all four were showing gross tremors and they were all observed to have generalized convulsions. The temperatures varied between 105·2 and 102·2° F.

On the next day (the seventh) three were moribund and the fourth was dead on the eighth day after inoculation. The brains were bacteriologically sterile and contained large quantities of virus.

Two three-day-old lambs, two seventeen-day-old and two hoggets were inoculated intradermally into the skin of the face with 0·25 ml. of the same material. Throughout the period of observation all these animals appeared well. Their temperatures varied between 104·0 and 100·2° F., quite irregularly, and four weeks after inoculation neither complement fixing nor neutralizing antibodies were present in the blood stream.

Horses.

A fifteen-year-old mare which showed no antibodies to the virus was inoculated intracerebrally with 1 ml. of 20 p.c. suspension of seventh mouse passage virus. On the ninth day after inoculation the animal showed slight ataxia and some jumpiness. On the tenth day the ataxia was markedly increased. The mare was bled on this day, but no virus was demonstrated by passage of heparinized blood to weaned mice. On the eleventh day the ataxia was very gross, but by the thirteenth day marked improvement was visible and nineteen days after the original inoculation no signs of illness, other than obvious loss of weight, were apparent. The animal developed complement fixing and neutralizing antibodies against the virus.

A nine-month-old foal was inoculated intradermally with 1 ml. of the same material. This animal appeared sick on the twenty-first day after inoculation, but showed no pyrexia and appeared quite well afterwards. She also developed specific complement fixing and neutralizing antibodies.

Monkeys (Macacus rhesus).

Two young rhesus monkeys were inoculated intracerebrally with 0·5 ml. of 20 p.c. fourth mouse passage brain material. One monkey began to show tremors on the sixth day after inoculation, was having violent convulsions on the seventh day and paralysed and moribund on the eighth. The other showed similar but less severe symptoms starting on the seventh day. It was hoped that this monkey would survive, but unfortunately it became moribund on the sixteenth day and was killed. Virus was present in large amounts in the brain of the monkey which died early, but was not demonstrated in that of the monkey dying late, although the neutralization index of the serum was only 1 log. The complement fixing titre was 1 : 320.

Brush Tailed Opossum (Trichosurus vulpecula).

Three recently trapped brush tailed opossums were inoculated intracerebrally with 0·3 ml. of 20 p.c. fifth passage material. The animals showed no signs of illness and thirty days after inoculation their serum contained neither

neutralizing nor complement fixing antibodies. There is no evidence that the virus was able to multiply in these animals.

Kangaroo (Macropus rufus).

A young male kangaroo received 0·5 ml. intracerebrally of 20 p.c. early mouse brain passage material. The animal showed no illness at any stage. One month after inoculation low titre complement fixing antibodies were present and two months after inoculation a neutralization index of 1·7 logs was found. No antibodies were present in the blood before inoculation.

Since a large dose of virus was inoculated the presence of this low titre of antibody cannot be regarded as conclusively proving that the virus had multiplied in this animal.

Chickens.

In experiments on chicks in down 14-day-old birds were used, because we had no satisfactory brooder accommodation in which younger birds could be kept. These birds showed large amounts of virus in the blood stream twenty-four hours after inoculation. At forty-eight hours after inoculation four out of four mice inoculated with heparinized blood died of the disease. At seventy-two hours three of four died and at ninety-six hours one of four. On succeeding days up to the ninth no virus was demonstrated in the blood. At this stage some of the birds were beginning to show marked leg weakness and one had already died. Deaths continued until the twenty-fourth day when the last had died. It is probable that most of these deaths were due to the virus. Birds inoculated intracerebrally and intramuscularly behaved in the same way.

Two healthy young cockerels were inoculated with 0·1 ml. of concentrated virus suspension intracerebrally. Neither bird showed any sign of illness at any stage. Virus was demonstrated in the blood stream of one at twenty-four and forty-eight hours. The other never showed sufficient virus in the blood to infect weaned mice by the intracerebral route. Both developed high titre neutralizing antibodies within one month of inoculation which remained high on subsequent testing. A complement fixation inhibition test showed inhibitory antibodies in the specimen taken at one month only.

Pigeons.

Three adult homing pigeons were inoculated intracerebrally and three intramuscularly with 0·1 ml. of 20 p.c. virus material. The birds were tested for the presence of virus in the blood at three day intervals up to the twelfth day. The only specimen showing the presence of virus was from pooled blood taken on the third day from the pigeons which received intracerebral virus. All the birds developed high titre antibodies.

Silver Gulls (Larus novo-hollandiae).

Two silver gulls were captured, one a first and the other a second-year bird. The birds ate readily in captivity. Both were inoculated intracerebrally with 0·1 ml. of 20 p.c. virus suspension. On the fourth day they began to look ill and by the sixth both were very ataxic. One died on this day, and the other was dead on the ninth morning. Both birds showed virus in the blood from twenty-four hours after inoculation until the last bleeding before death (on the sixth and eighth days respectively). The mice inoculated with blood taken on days one to five died on the fifth or sixth day after inoculation. Those receiving sixth-day blood died on the seventh or eighth. Only one of four mice receiving blood taken on the eighth day died, but showed the typical signs of infection with this virus.

It may be of importance that the virus is so much more easily demonstrated in the blood of the birds of this species than in that of any of the other animals we have tested.

Ducks.

Two adult domestic ducks were inoculated intracerebrally with 0·1 ml. of 20 p.c. virus suspension. Virus, which had been absent from the blood stream of both birds six hours after inoculation, was present in specimens taken at twenty-four, forty-eight and seventy-two hours in sufficient quantity to kill at least three out of four weaned mice inoculated intracerebrally with 0·03 ml. of heparinized blood. On the fourth day one bird was found dead and the other was markedly ataxic. One of four mice inoculated with fourth-day blood from the survivor died. Virus was not demonstrated in the blood later than this and the bird had recovered completely by the tenth day after inoculation. Neither bird was feeding well during the first four days after inoculation.

Embryonated hen's egg.

The embryonated hen's egg is highly susceptible to inoculation with the virus by the yolk sac route. When large doses of virus are inoculated into eggs incubated between six and nine days the embryos usually die forty-eight hours after inoculation and are always dead by seventy-two hours after inoculation. The titre of the virus when inoculated in 0·25 ml. quantities into the yolk sac of embryonated hen's eggs after six days' incubation was log 8·6. The virus did not fade out during yolk sac passage.

The virus produced very small opaque pocks on the chorioallantoic membrane of embryonated hen's eggs inoculated on either the tenth or twelfth day of incubation. Numerous pocks were found after direct inoculation of concentrated mouse brain material, but in our hands passage on the chorioallantoic membrane was difficult and the virus rapidly faded out when passed by this route.

Thermal death point.

1 ml. volumes of 20 p.c. mouse brain suspension in 10 p.c. rabbit serum saline were heated in thin walled test tubes in a water bath to the temperatures and for the times shown in Table 4. Virus was still active after thirty minutes heating at 51° C., but mice died one day later than the controls receiving unheated virus. Virus heated to 56° C. for fifteen minutes was inactive as tested intracerebrally in weaned mice.

TABLE 4.

Thermal death point.

Treatment of virus	Number of mice in group	Day of mouse death
Heated 51° C. 30 minutes	4	6, 6, 6, 6
Heated 56° C. 15 minutes	4	s, s, s, s
Heated 56° C. 30 minutes	4	s, s, s, s
Heated 70° C. 15 minutes	4	s, s, s, s
Unheated	4	5, 5, 5, 5

Filtration.

A series of experiments was conducted to make an estimate of the particle size of the virus. The original 20 p.c. serum saline suspension of virus was diluted to one per cent. in saline and immediately filtered through gradocol membranes. Before some of the filtrations with fine pore membranes preliminary filtration at a larger pore was carried out, but this did not lead to the expected improvement in the ease with which the virus passed through the fine membranes. Virus passed through membranes having an Average Pore Diameter (APD) of 120 mμ and in one of four experiments a minimal amount of virus passed through a filter with a 100 mμ APD. Virus did not pass through membranes having an APD of 60 or 55 mμ. Membranes between 100 mμ and 60 mμ APD were not available, but the evidence presented strongly suggests that the 100 mμ closely approached the APD at which the virus would be completely held back and suggests a probable particle size of 20–50 mμ (Elford, 1933).

Histology.

The histological appearances of the brain from the human case which was the source of this strain of virus has already been described (Miles, Fowler and Howes, 1951). The slightness of the cellular reaction noticeable in this material was also obvious in the experimental animals infected with the virus, but otherwise the appearances were very similar to those given by Cleland and Campbell (1919) and by Perdrau (1936) for X-disease and that given by Robertson for the recent outbreak of encephalitis in the Murray Valley.

TABLE 5.

Filtration through gradocol membranes.

Average pore diameter of membranes		
Preliminary filtration	Final filtration	Mouse deaths
—	750 mμ	4/4
—	600 mμ	4/4
—	400 mμ	4/4
—	200 mμ	4/4
—	200 mμ	2/4
400 mμ	200 mμ	0/4
—	190 mμ	4/4
750 mμ	190 mμ	0/4
—	120 mμ	4/4
750 mμ	120 mμ	0/4
—	100 mμ	*1/4
—	100 mμ	0/4
600 mμ	100 mμ	0/4
400 mμ	100 mμ	0/4
—	60 mμ	0/4
600 mμ	60 mμ	0/4
—	55 mμ	0/4

Numerator = Mouse deaths. Denominator = Number of mice in group.

* Passage of the brain from this mouse confirmed that this death was due to the virus.

DISCUSSION.

The main problem arising from the description of those characteristics of the virus which have been discussed in this paper is whether or not it is the same as the virus responsible for the outbreaks of X-disease described by Cleland and his associates.

No strain of X-virus is available for comparison and we can only compare the results of inoculations in the range of experimental animals which both Cleland and Campbell and ourselves have used.

In both series rhesus monkeys, horses and sheep were susceptible to intracerebral inoculation and the disease produced was essentially the same in each of these species in both series. In both series rabbits, guinea-pigs and adult fowls were not found to show any clinical disease, although in our series a transient pyrexia was regularly found in the guinea-pigs and viremia was shown to be present in one cockerel for forty-eight hours. In view of these similarities, despite the slight differences in the histological picture to which reference has been made, we think that it is highly probable that this virus is identical with that causing the outbreaks of Australian X-disease.

Suggestions have been made that X-virus might be closely related to that of Louping-Ill (Burnet, 1934 and Perdrau, 1936) but the incubation period in mice infected with Louping Ill is longer than with this virus when the same strain of mice is used; further, Louping Ill virus does not kill chick embryos so rapidly

after yolk sac inoculation as does this virus and in our hands this virus has not been found to cause the enormous haemorrhagic oedema in the chick embryo which we have regularly observed in embryos infected with Louping Ill.

The other virus to which this shows great similarity is Japanese B. The majority of workers appear to have found the rabbit susceptible to infection with Japanese B., but there have been some exceptions. We have never even succeeded in showing pyrexia in rabbits inoculated with our virus. Otherwise the pathogenicity spectrum of the two viruses is very similar and a relationship to the Japanese B. group seems probable.

SUMMARY.

A virus has been isolated from a fatal human case of encephalitis occurring in South Australia.

Monkeys, mice, horses, lambs, silver gulls, suckling rats and young chicks have been shown to develop clinical disease after intracerebral inoculation with the virus.

In rabbits, guinea-pigs, weaned rats, pigeons and adult fowls no clinical disease has been observed, but the evidence suggests that the virus can multiply in these animals.

Brush tailed opossums and a kangaroo appeared to be completely insusceptible and sheep were insusceptible to peripheral inoculation.

The embryonated hen's egg was susceptible to inoculation by the yolk sac and the chorio-allantoic routes.

The virus survived heating to 51° C. for thirty minutes, but not heating to 56° C. for fifteen minutes.

Filtration experiments suggest a particle size for the virus between 20–50 mμ.

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AN ENCEPHALITIS VIRUS ISOLATED IN SOUTH AUSTRALIA

II. SEROLOGICAL OBSERVATIONS ON THE VIRUS AND ITS RELATION TO OTHER ARTHROPOD-BORNE ENCEPHALITIDES.

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In the previous paper observations on a strain of encephalitis virus isolated from the brain of a fatal case of encephalitis in a child have been described. In this paper we describe the development of serological tests using this virus, the application of these tests to the investigation of human and animal sera, the course of antibody production in experimental animals and the results of such limited investigations as we have been able to make on the serological relations of our strain with other encephalitis viruses.

MATERIALS AND METHODS.

Strains of Virus.

The South Australian virus was used after 15–20 passages in mouse-brain for the preparation of mouse brain antigens. For the preparation of chick embryo antigens it was used between the first and fifth yolk sac passage.

The Murray Valley encephalitis virus was obtained from the Walter and Eliza Hall Institute through the kindness of Sir Macfarlane Burnet and Mr. E. L. French.

The St. Louis virus was from a dried preparation available at this Institute. Its previous history is unknown. It was neutralized to titre by an antiserum against St. Louis virus.

Prepared complement fixing antigens from Japanese B., St. Louis, West Nile and Western equine encephalitis viruses were obtained from the Virus Division of Lederle Laboratories, Pearl River, New York, through the kindness of Dr. Hilary Koprowski.

Antisera.

Experimental antisera were obtained by a single intracerebral inoculation of a moderate dose of mouse passage virus. By this technique high titre complement fixing and neutralizing antibodies could be obtained in guinea-pigs and rabbits.

A high titre complement fixing antiserum containing little neutralizing antibody, was obtained from a monkey dying on the sixteenth day after intracerebral inoculation.

Dr. K. C. Smithburn, of the Laboratories of the Rockefeller Foundation for Medical Research, New York, kindly supplied monkey neutralizing antisera against Japanese B., St. Louis, West Nile, Russian Spring Summer, Western equine and Mengo encephalitis viruses, Dr. A. B. Sabin of Cincinnati a rabbit antiserum against Japanese B., Mr. E. L.

[1]Assisted by grant from the National Health and Medical Research Council of Australia.

French a monkey antiserum against Murray Valley encephalitis and Dr. M. C. Fowler a guinea-pig antiserum against *Herpes febrilis*.

Neutralization tests.

In the neutralization tests, equal volumes of undiluted serum were mixed with dilutions of virus with penicillin and streptomycin added to give 500 units of each antibiotic per ml. The mixtures were incubated at 37° C. for two hours and, if they could not be used immediately, were stored at 0° C. for periods up to one hour. Normal rabbit serum was used for the control virus dilutions. All sera were inactivated for thirty minutes at 56° C. before use. The activity of the mixtures was tested intracerebrally in weaned mice between the ages of twenty-one and twenty-eight days.

Where the neutralization indices of the sera were to be determined, groups of five mice were used for each dilution. In tests for the presence or absence of significant amounts of antibody, ordinarily one group of six mice was used, the test mixture containing an estimated 75 LD_{50} of virus. The actual amount present varied, but was seldom below 50 or above 150. When antibody capable of neutralizing 50 LD_{50} or more of virus was present the serum was regarded as positive. On the few occasions when insufficient virus was present in the mixture to determine this, the test was repeated in all sera giving a doubtful positive result. Negative results from such mixtures were not repeated.

The Neutralization Index of the serum specimens is expressed as the log of the number of LD_{50} of virus neutralized by the given specimens of serum, under the conditions described.

Complement fixation test.

1. *Preparation of antigens.*

A. *Chick embryo antigen.* Embryonated hens' eggs, on the ninth day of incubation, were inoculated into the yolk sac with 0·1 ml. of a 1–2 p.c. infected mouse-brain or chick embryo suspension. Forty-eight hours after inoculation, all embryos, whether dead, or alive, were harvested. The embryos were beheaded and the bodies were ground with silica in a mortar, and a 20 p.c. suspension in isotonic saline was prepared. After overnight extraction at 4° C., the crude suspension was centrifuged at 5,000 r.p.m. for two hours in an angle centrifuge kept at 4° C. The supernatant was then dispensed in 4 ml. lots and kept at —15° C. until use.

Control antigen was prepared from normal eleventh day embryos in a manner identical to the above.

B. *Mouse-brain antigen.* Antigens were prepared by the method developed by Espana and Hammon (1947). Three to four weeks' old mice were inoculated by the intracerebral route with 0·03 ml. of a 1–2 p.c. infected mouse-brain suspension, and four to five days later, when moribund, they were bled out by section of the brachial artery. The brains were then removed and a 20 p.c. suspension in distilled water was prepared by grinding in a mortar with silica. Following overnight extraction at 4° C. the suspension was lyophilized.

The dried preparation was extracted for two hours with approximately twice its original volume of benzene, and then filtered through a scintered glass funnel at the filter pump. After two washings with benzene, the extracted product was resuspended in its original volume of saline and left overnight at 4° C. Finally the suspension was centrifuged at 5,000 r.p.m. at 4° C. for two hours, and the supernatant, after the addition of merthiolate (1: 10,000), dispensed in 1 oz. McCartney bottles and kept at 4° C. until use. With later batches, the dry benzene extracted product was resuspended in distilled water, centrifuged and 4 ml. quantities lyophilized and stored at 4° C.

Control antigen was prepared from normal mice of the same age as those used in the preparation of the virus antigen by the same procedure.

2. *Technique of the complement fixation test.*

Sera. All sera used in the tests were inactivated at 56° C. for thirty minutes before use. When sera had previously been inactivated and stored, inactivation was repeated for a further ten minutes.

Antigens. The Lederle antigens in these tests were used at a 1: 8 dilution. Mouse-brain antigens prepared in the laboratory were used at a 1: 8 dilution, with the exception of one batch which was used at a 1: 4 dilution. Chick embryo antigens were used undiluted.

Complement. Guinea-pig complement stored at —15° C. or, after lyophilization, at 4° C. was titrated alone and in the presence of antigens. Two M.H.D. of complement were used in the tests.

Sensitized red cells. Three p.c. sheep red cells sensitized with 5 M.H.D. haemolysin were used.

Technique. A four volume technique was used throughout. Dilutions of all reagents used in the test were carried out in calcium magnesium saline. Fixation was allowed to take place at 4° C. overnight and, after addition of sensitized red cells, racks were placed in a 37° C. water bath for thirty minutes.

EXPERIMENTAL.

Complement fixation test.

Complement fixing antigens were prepared as described in the section on methods. In our earliest experiments we used a complement fixing antigen prepared by simple saline extraction of beheaded embryos This antigen usually showed specific activity to a dilution of 1:4 or 1:8, but the results, especially with animal sera, were sometimes bedevilled by quite high titre fixation with normal embryo extracts prepared in the same way (see Table 1).

We, therefore, decided to try to prepare antigens from lyophilized, benzene extracted chick embryo and mouse-brain, following the technique of Espana and Hammon. Normally, specific fixation with the chick embryo extract could only be obtained to an antigen dilution of 1:8, while mouse-brain extract was usually active to 1:32. Both antigens, when used at a concentration giving optimal fixation, gave similar titres with the same sera, but because of the higher titre of the mouse-brain preparations, such preparations were used in all but the earliest experiments.

A series of aboriginal sera, among which a high percentage showed positive Wassermann and Kahn reactions, also contained a number of sera giving positive complement fixation tests with our benzene extracted antigen. In no case was there any correspondence between the W.R. positive and encephalitis positive sera. Table 2 gives the results of ten of these tests in detail. The Table also includes results of complement fixation tests for psittacosis on the same sera, showing merely chance overlapping in one serum positive both to encephalitis and psittacosis antigens. In all we tested, thirteen sera were positive and ten doubtful to the Wassermann, D.K. and Kahn tests. None of these gave a positive reaction with our benzene extracted mouse-brain antigen.

We did not find any positive C.F.Ts. in the sera of laboratory animals before they had been bled, nor did we find positive human sera among numerous specimens from the Adelaide metropolitan area.

TABLE 1.

Comparison of complement fixation test results using various antigens.

Antigen	Serum (Exp. 1) Monkey	Japanese B. Rabbit	Rabbit 6	Guinea-pigs 3 & 4	Horse	Human 1	Human 2	Human 3
Normal egg S.A. virus	1:5	1:320	1:10	0	NT	0	0	NT
Infected egg	1:160	1:640	1:160	1:80	NT	1:80	1:80	NT
*Japanese B.	1:80	1:80	1:40	1:10	NT	1:5	±1:5	NT
*St. Louis	1:10	1:40	1:20	±1:5	NT	0	0	NT
*West. Equine	0	NT	1:10	±1:5	NT	0	0	NT
Serum control	0	0	0	0	NT	0	0	NT
	(Exp. II)							
Mouse-brain S.A. virus	0	1:40	0	0	0	0	0	0
Infected Mouse-brain	1:320	1:80	1:160	1:40	1:20	1:80	1:40	1:5
*Japanese B.	1:40	1:80	1:40	1:20	0	1:5	0	0
*St. Louis	1:10	1:40	1:40	1:5	0	0	0	0
*West. Equine	±1:5	1:40	1:10	0	0	0	0	0
Serum control	0	0	0	0	0	0	0	0

NT—Not tested.

* These antigens were supplied by Lederle Laboratories Division, Pearl River, N.Y.

Table 1 shows the results of testing the sera of animals experimentally infected with the virus and three sera from human cases against crude egg and benzene extracted mouse-brain S.A. virus antigens and against commercial Japanese B., St. Louis and Western equine encephalitis antigens. The Japanese B. rabbit serum gave so much non-specific crossing that the results are of little value. The rabbit, guinea-pig and monkey sera against our virus showed substantial crossing with Japanese B. and St. Louis, and the rabbit serum gave a low titre reaction with Western equine encephalomyelitis, but in each case the titre against the homologous virus was much greater than that against the heterologous. The horse serum and two human sera showed no crossing with any other virus. The third human serum gave fixation to a titre of 1:80 with the homologous virus and a titre of 1:5 against Japanese B.

There was no significant difference in positive titres whether chick embryo or mouse-brain antigens were used.

The results strongly suggest that the mouse-brain antigen is more highly specific and in other ways quite as satisfactory as the chick embryo antigen. The monkey, rabbit and guinea-pig sera suggest a relationship to Japanese B. and St. Louis encephalitides, while the human and horse sera indicate that our virus is sharply distinct from these possibly related viruses.

TABLE 2.

Specificity of the complement fixation and neutralization tests in certain human sera.

Serum No.	Neutralization	Encephalitis C.F.T.	W.R.	D.K.	Kahn	Psittacosis C.F.T.
C.A. 2	—ve.	—ve.	++	+++	++	—ve.
C.A. 3	—ve.	—ve.	—ve.	—ve.	—ve.	1:8
C.A. 10	*+ve.	1:20	—ve.	—ve.	—ve.	NT
C.A. 17	+ve.	—ve.	—ve.	—ve.	NT	NT
C.A. 19	+ve.	—ve.	—ve.	—ve.	—ve.	1:4
C.A. 22	—ve.	—ve.	++++	++++	+++	—ve.
C.A. 26	—ve.	—ve.	++++	++++	++++	—ve.
C.A. 28	+ve.	1:5	—ve.	—ve.	NT	—ve.
C.A. 31	—ve.	—ve.	++	++++	++++	1:8
C.A. 36	+ve.	1:10	—ve.	—ve.	NT	—ve.

NT—Not tested.

* A positive neutralization is recorded where the neutralization index of the serum is over 50.

Neutralization tests.

The technique for the neutralization tests which was described in the methods section was used throughout this series of experiments. Where single tests were performed, only sera having a neutralization index of 1·7 logs or higher were recorded as having antibodies against the virus. No laboratory animals were shown to have any neutralizing antibodies before inoculation and the horses used experimentally were shown to have neither complement fixing nor neutralizing antibodies before they were inoculated.

TABLE 3.

Neutralization with standard sera.

Antiserum prepared against	Animal used	Neutralization index with S.A.-virus (mean of two tests)	Neutralization index with homologous and related-viruses
S.A. virus	Rabbit	3·6	†3·6
Mengo	Monkey	0	*4·7
Russian Spring Summer	Monkey	0	*3·6 (†with Louping Ill 2·0)
St. Louis	Monkey	0	*3·9
West Nile	Monkey	0	*2·7
Japanese B.	Monkey	0·5	*2·8
Japanese B.	Rabbit	2·3	**6·1 (with St. Louis 2·7) (with West Nile 2·3)

* Tested by Dr. K. C. Smithburn. ** Tested by Dr. A. B. Sabin. † Tested by us.

Neutralizations were carried out using standard antisera against a variety of the arthropod-borne encephalitides. The results of these tests are shown in Table 3. Monkey sera did not give any definite evidence of relationship

between the S.A. virus and Mengo, Russian Spring Summer or viruses of the Japanese B. group, but a rabbit serum against Japanese B. indicated that a relationship did exist. The neutralization of the S.A. virus was of the same order as that given with St. Louis and West Nile viruses by the same serum. A guinea-pig serum prepared against *Herpes febrilis* virus had no neutralizing action against our virus. These results confirm our findings with the complement fixation test, which indicated that the S.A. virus had some relationship to the Japanese B. group, but that it was quite markedly different from both Japanese B. and St. Louis viruses.

Cross neutralization.

We had no access to a wide variety of strains of encephalitis virus, but we have been able to carry out cross neutralization tests using S.A. virus, Murray Valley encephalitis, St. Louis encephalitis and Louping Ill (Table 4). The results indicate that this virus is identical with the Murray Valley virus and sera prepared against both these viruses neutralized St. Louis virus to a significant extent. However, in two experiments guinea-pig sera against St. Louis virus failed to neutralize either M.V.E. or S.A. although the neutralization index of one specimen for the homologous virus was 1·8 logs and of the other 3·0 logs. There was no evidence of cross neutralization with Louping Ill virus.

TABLE 4.

Cross Neutralization.
Log of neutralization indices.

	Serum				
Virus	S.A. Guinea-pig 5	S.A. Guinea-pig 7	M.V.E. Monkey K 3	St. Louis G–p 1 and 2	Louping Ill Rats
S.A.	3·5	4·6	3·3	*a 0 **b 0	0·5
M.V.E.	3·9	NT	3·5	a 0·1 b NT	NT
St. Louis	2·4	2·1	1·8	a 1·8 b 3·0	NT
Louping Ill	—1·0	NT	NT	NT	2·8

* Specimen of serum taken 5 weeks after inoculation.
** Specimen of serum taken 9 weeks after inoculation.
NT—Not tested.

These results are not complete because of our lack of a full range of virus strains, but they justify the conclusion that these Australian strains of virus should be regarded as representing a fourth member of the Japanese B. group.

Development of antibodies.

The development and persistence of complement fixing and neutralizing antibodies have been followed in three guinea-pigs inoculated with virus intracerebrally and in two horses, one inoculated intracerebrally and the other intradermally.

None of the guinea-pigs showed any complement fixing antibodies seven days after inoculation, but fourteen days after, two had reached maximum titre, while the third, which showed a titre of 1 : 20 at the fourteenth day after inoculation, gave a titre of 1 : 40 by the twenty-first day (Table 5). One animal kept a very steady titre from the twenty-first day until it died more than two hundred and three days after inoculation. The titre in the second showed some evidence of falling when the animal was last tested one hundred and twenty-three days after inoculation and the third was also showing a reduced titre from about the ninety-fifth day until the experiment was terminated on the two hundred and seventy-seventh day after inoculation. Even at this late stage the complement fixing titre was 1 : 20.

TABLE 5.

Neutralization and complement fixation tests on guinea-pig sera following intracerebral inoculation with S.A. virus.

Days after inoculation	Guinea-pig 5		Guinea-pig 7		Guinea-pig 8	
	C.F. titre	Log Neutralization index	C.F. titre	Log Neutralization index	C.F. titre	Log Neutralization index
0	0	0	0	0	0	0
7	0	1·1	0	NT	0	NT
19	1:80	1·2	1:40	NT	1:20	NT
21	1:80	1·0	1:40	1·8	1:40	1·3
28	1:80	2·7	1:20	2·2	1:40	2·5
35	1:80	4·2	1:40	3·1	1:40	2·9
67	1:40	4·9	1:20	3·6	1:40	3·1
95	1:20	4·2	1:10	4·7	1:40	2·7
123	1:40	2·9	1:20	4·57	1:40	3·7
166	1:10	4·7		Died	1:40	3·0
203	1:40	3·9			1:80	3·1
242	1:10	3·1				Died
277	1:20	3·1				

The course of the neutralizing antibodies was substantially different. Only one animal showed a significant titre on the twenty-first day after inoculation, but all had reached a significant level by the twenty-eighth day. However, peak levels were only reached two to three months after inoculation. The only animal in which there was any evidence of a fall in titre before the end of the experiment was that which survived two hundred and seventy-seven days. The last two readings on this animal showed a neutralization index 1·8 logs below the highest recorded sixty-seven days after inoculation.

The early development of the antibodies was not followed closely in the horses. The animal inoculated intracerebrally developed overt signs of the disease (as described in the previous paper). Twenty-seven days after inoculation this animal showed a complement fixing titre of 1:20, but no significant amount of neutralizing antibody. The neutralization index had risen to 2·7 logs by the sixtieth day, but by this time complement fixing antibodies had disappeared. From then until the one hundred and sixty-seventh day the neutralization index showed only chance variation, but positive complement fixation was not found. In the other animal low titre complement fixing antibodies were found from the sixty-third to the one hundred and fifty-eighth day after inoculation. They were absent on the two hundred and fifth day after inoculation. There was a suggestive, but not significant, fall in the neutralization index in the two hundred and fifth day specimen, the results from all previous bleedings having been remarkably steady.

TABLE 6.

Neutralization and complement fixation tests on horse sera following intracerebral and peripheral inoculation with S.A. virus.

Inoculated intracerebrally			Inoculated subcutaneously		
Days after inoculation	C.F. titre	Neutralization index	Days after inoculation	C.F. titre	Neutralization index
0	0	0	0	0	0
27	1:20	1·0	63	1:10	3·0
60	0	2·7	88	1:5	2·9
88	0	2·7	116	1:5	2·8
130	0	2·1	158	1:10	2·9
167	0	2·2	205	0	1·9

These results show in both species a relatively rapid rise in complement fixing antibody titre, while the development of neutralizing antibodies is delayed. In the guinea-pig both antibodies are long lasting, but in the horses tested the complement fixing antibody was only transiently present, while neutralizing antibody appeared to be long lasting.

Results with sera collected in the field.

The results of complement fixation and neutralization tests on human and horse sera collected in various areas of South Australia and the Northern Territory are shown in Table 7. In each group there were approximately twice as many positive sera by the neutralization test as by the complement fixation test. If both groups are considered together, in three hundred and seventy tests, fifty-four positive neutralizations (neutralization index 1·7 logs) were found as against twenty-six positive complement fixation tests (complete fixation of two units of complement at a serum dilution of 1:5 or higher). Twenty-two sera were positive to both tests and only four sera showing a positive complement fixation test failed to give a positive neutralization.

Thirty-two sera were positive to the neutralization test only. The total number positive either to one or to both tests was fifty-eight.

TABLE 7.

Results of serological tests on human and horse sera collected in the field.

Serum	No. of cases tested	Positive neutralization test	Positive complement fixation test	Positive to both tests	Positive neutralization only	Positive complement fixation only
Human	284	25	12	9	16	3
Horse	86	29	14	13	16	1

DISCUSSION.

The complement fixation results using antigens from our virus and from Japanese B., St. Louis and Western equine viruses apparently show a very high specificity in the horse and human sera, only one of which showed any crossing with any virus other than that known or suspected to be the cause of the infection. The rodent and monkey sera showed a broader activity, all showing fixation with both Japanese B. and St. Louis antigens, though to a lower titre than with the homologous antigen. The rabbit serum also showed definite low titre crossing with Western equine encephalitis despite showing no activity against normal mouse-brain. However, such low non-specific titres are not uncommonly seen particularly with rodent sera. The substantially higher titres and consistently positive results with the guinea-pig, rabbit and monkey sera with Japanese B. and St. Louis antigens are strong evidence that our virus is related to this group.

The results of the neutralization tests confirm this. There was no significant degree of neutralization of our virus by Dr. Smithburn's highly specific sera against all the viruses of the Japanese B. group, but Dr. Sabin's rabbit serum against Japanese B. did neutralize the S.A. virus to a significant extent, although 3·8 logs lower than its titre with the homologous virus. Our cross neutralization tests also indicated a relation to St. Louis virus, but certainly not an identity between the two viruses. The S.A. virus and the Murray Valley encephalitis viruses appear to be identical. We suggest that they should be regarded as a further member of the Japanese B. group. French (1952) has pointed out a relation between M.V.E. and Japanese B.

The development of antibodies against these two viruses in guinea-pigs and horses follows a course similar to that for Japanese B. and St. Louis viruses in man (Hammon, 1948). The complement fixing antibody appeared before the neutralizing antibody and in the horse, at any rate, disappeared quite rapidly. Anderson *et al.* (1952) have given evidence to suggest that the complement fixing antibody in man, particularly following inapparent immun-

izing infections during the Murray Valley epidemic, might fall off quite rapidly.

Our results comparing the complement fixation and neutralization tests on human and horse sera collected in the field, confirm the impression that the neutralizing antibody remains much longer than the complement fixing. We are inclined to think that the presence of complement fixing antibodies normally indicates a fairly recent infection, probably within the year, and that the presence of neutralizing antibodies normally suggests an infection occurring three weeks or more previously. This antibody may be long lasting. We think that there are considerable advantages in using both tests in a serological survey.

The Melbourne workers (Burnet, 1952) have named the virus on which both they and we have been working "Murray Valley encephalitis" and this name appears to be becoming generally accepted, but we think that we are, in all probability, dealing with a recurrence of Australian X disease.

SUMMARY.

Serological investigations on a strain of encephalitis virus isolated from a fatal case in South Australia are described.

Evidence is produced that this virus is related to members of the Japanese B. group of viruses, but not identical with any of the previously recognized members.

Evidence is given to show that it is identical with the Murray Valley encephalitis virus.

Evidence is produced that in the guinea-pig and the horse, complement fixing antibodies to this virus are more rapidly produced than neutralizing antibodies and that in the horse neutralizing antibodies are more enduring than complement fixing antibodies.

Field investigations on human and horse sera show twice as many positive neutralization as complement fixation tests. This difference is likely to be due to the more enduring character of the neutralizing antibody.

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